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EXPERIMENTAL IMMUNOCHEMISTRY

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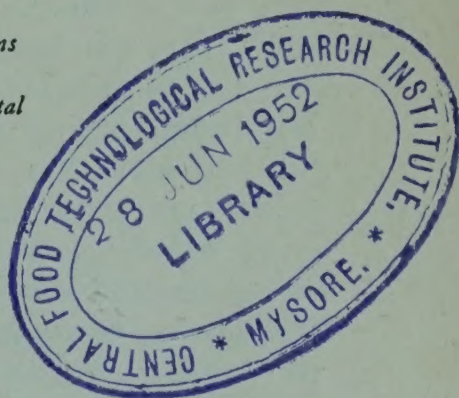
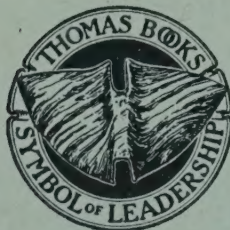
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PREVIEW

Had the writer not undertaken this preliminary note he would undoubtedly have been asked to review "Experimental Immunochemistry": hence the title of this Introduction. This would seem the more appropriate since he has, though not licensed to practice medicine, functioned as obstetrician to this book: without going into unseemly details he was present at its inception, watched and aided its gestation, and successfully avoided paternity (in another affair) by depositing this lusty infant in the arms of a willing and gently persistent publisher. Scandalous obstetrics, undoubtedly, but now that authors and publisher are happily engaged in mopping up the final details of midwifery, the "doctor" may sit back in an armchair, wipe his brow, and dispassionately survey what has been produced.

There have been few books on immunochemistry, for its rapid and vigorous experimental growth in many directions has not provided the quiet, contemplative atmosphere essential for writing. Arrhenius' little book of provocative lectures was the first, and Wells' *The Chemical Aspects of Immunity* in its two editions, provided a thorough survey up to the period in which the impact of new quantitative, absolute methods was beginning to be felt. The three editions of Landsteiner's *Specificity of Serological Reactions* (including the initial German version), widely spaced as they were, reflect the rapid growth of the subject although the main theme was treated essentially from the qualitative standpoint and has only recently been approached quantitatively. The two editions of Marrack's monograph, *The Chemistry of Antigens and Antibodies*, with their logic of presentation, tempered by stimulating speculation, filled a valuable purpose, and the speed with which they went out of print was an indication of their utility. Boyd's *Fundamentals of Immunology* was broader in scope and also appears to have filled a definite need. It is in part an immunochemistry, written from a point of view very different from that of Kabat and Mayer. Sevag's *Immunocatalysis* is a specialized treatise dealing with only a portion of the broad field and emphasizing the analogies between enzymatic and immune reactions.

From this brief survey it becomes evident that a complete *Im-*

munochemistry is yet to be written. The book under preview does not attempt to fill this gap, but its authors have undertaken the major part of the task by assembling, for the first time in one place, the scattered techniques which have been developed to fill the needs of immunochemical problems as they unfolded, so that knowledge in this complex and widely inclusive field might be advanced. These methods, drawn from physics, physical chemistry, analytical chemistry, and organic chemistry and from the biological sciences as well, are described in their working details and discussed as to their applicability and limitations. The book is, therefore, more likely to gather acid spots and indicator stains on the laboratory table than to accumulate dust on the reference shelves.

The great potency of the immunochemist's methods is even now incompletely realized. The immunochemist is in possession of a store of marked molecules, antigens and antibodies, each as distinctively marked with respect to the other as if it contained a radioactive tracer element or an excess of an isotope. The comparison with the materials and methods of the atomic physicist is valid in an additional respect. Just as an isotope of carbon, hydrogen, or nitrogen may be followed through the most complex metabolic or pathological changes by means of a sensitive and rigorous analysis in the mass spectrograph, or a few radioactive phosphorus atoms may be traced even more sensitively by their inevitable activity, so may antigens and antibodies be made to interact and that interaction measured quantitatively with all the rigorous precision of which analytical chemistry is capable. Kabat and Mayer, in placing their main emphasis on these quantitative analytical methods, bring out anew some of the important services these methods are capable of rendering.

In Part I of the book the authors plunge rapidly into complete and usable descriptions of the principal methods for the study of the substances and reactions with which immunology is concerned. The five sections of Part II describe in detail the application of quantitative immunochemical methods to actual problems, many of which one or the other of the authors took part in solving. This portion of the book should be of great value in suggesting to the reader the extent to which the methods laid before him might be of use in helping solve questions with which he is preoccupied. Not the least service rendered here by the authors is their exposure of

the limitations of the methods they describe, so that no one should be tempted to exploit them beyond their scope.

Part III of the book provides working instructions for numerous chemical and physical procedures which are the stock in trade of immunochemists, while Part IV deals with the preparation of well characterized individual proteins, enzymes, specific polysaccharides, and related materials. Much of the matter in these divisions, as well, is from the authors' own experience. The book closes with an Appendix dealing with miscellaneous items of equipment and technique which should, in one way or another, be of assistance especially to less experienced workers.

Anyone who has read this far will know whether or not "Experimental Immunochemistry" will serve his ends. The writer feels certain that many workers will proceed into the book itself.

MICHAEL HEIDELBERGER

P R E F A C E

In this book we have endeavored to make available details of the experimental methods used by the immunochemist, together with an exposition of the basic principles and operational rationale of immunochemistry. As a borderline science, immunochemistry offers an approach to many chemical and biological problems and furnishes highly specific and precise quantitative methods for the study of proteins and of certain carbohydrates. It has proven of value as a guide in the purification of many substances from such complexes as bacteria, serum and plasma, and of tissue constituents. At the present time, however, there does not exist, except for a few reviews, any comprehensive work by which individuals interested in the applications of immunochemistry to research or teaching may become familiar with its rationale and procedures. Most of the methods have been developed and modified continuously during the past two decades and are scattered in numerous publications through various scientific journals. It is the hope of the writers that their attempt to outline the methodology of immunochemistry, with special emphasis on the applications and uses of quantitative chemical techniques as developed largely through the work of Drs. Michael Heidelberger and Forrest E. Kendall, may serve as an introduction to experimental immunochemistry.

To meet the needs of students as well as of chemists who may not be thoroughly familiar with immunology, and of bacteriologists and immunologists who may not be well acquainted with microchemical methods, introductory and background material in both fields has been included. Some of this may seem elementary to the specialist. For example, details of dilution methods for the estimation of antibodies may prove of value to the chemist and of little use to the serologist. Similarly, several of the chemical methods may assist the latter group, although the chemist uses them routinely.

As this is essentially a book on experimental methods and their use, little or no consideration has been given to controversial subjects such as mechanisms of immune reactions, theories of formation of antigen and antibody, and the nature of the forces between antigen and antibody, but references to work on these subjects are included. In line with this, no discussion is given of the role of his-

tamine in allergic reactions, and only the underlying antigen-antibody reaction is considered. In this, as well as in other respects, the book is not to be considered a comprehensive textbook embodying all of immunochemistry. It is essentially intended to be an exposition of the experimental aspects of the subject.

Many highly specialized phases of immunology have not been considered in detail. For example, the procedure for blood grouping is given chiefly to illustrate a widely used slide agglutination technique, and is not intended as a complete or even adequate consideration of this field.

The choice and arrangement of the subject matter have been dictated by operational considerations. Parts I and II contain a detailed treatment of immunological and immunochemical methods and their applications, with emphasis on the evaluation of results by the quantitative methods. In Part III are described a variety of chemical and physical methods and special procedures frequently used by the immunochemist. While the authors realize that the costly equipment required for electrophoretic, ultracentrifugal, and diffusion studies is not accessible to a large number of workers, they nevertheless believe that inclusion of the sections may be helpful to the understanding and evaluation of the many papers on these subjects which are constantly appearing in the literature. Part IV includes details for preparing a variety of substances of importance in immunochemical work. Most of the preparations given in Part IV have been successfully used in at least two different laboratories and they have been chosen to illustrate a variety of techniques for isolation and purification. The materials selected represent a group of well defined substances of immunochemical interest. Since the authors believe that results of far greater significance can be obtained with well-defined chemical entities, rather than with mixtures of proteins such as whole serum, milk, crude bacterial extracts, etc., they have only drawn upon studies with mixtures of antigens for background material.

The order in which the book is read will depend largely upon the background and interests of the reader. For those not familiar with modern physicochemical studies, it is suggested that the sections in Part III on electrophoretic and ultracentrifugal analysis and diffusion, and the section in Part IV on purification of antibodies be

read before Chapter 6 of Part I on antibodies and their characterization.

We are especially indebted to the Journal of Experimental Medicine and to the Journal of Immunology, from which much material has been drawn; to Professor T. Svedberg and Dr. K. O. Pedersen and to the Clarendon Press, Oxford, for permission to reproduce material from *The Ultracentrifuge*, and to numerous other authors, publishers and journals as indicated in the text.

It is a pleasure to acknowledge the many helpful comments and suggestions of Drs. A. Bendich, T. Rosebury, B. C. Seegal, and A. J. Weil about various portions of the manuscript, and the aid of Miss Helen Detweiler, Miss Virginia Fitzgerald, and Mrs. Jeannette Molter, in typing the manuscript. We are deeply indebted to Professor Michael Heidelberger for his continued interest, inspiration and criticism, without which this book would not have been possible.

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New York City

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Part I

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CHAPTER I

INTRODUCTION

Immunochemistry, the study of the chemistry of antigens and antibodies and of the chemical basis of immunity and resistance to disease, developed from immunology and, in general, still employs its methods and nomenclature. It has, however, of recent years become a discipline in its own right due largely to the development of precise analytical chemical methods for measurement of both antigens and antibodies and because of the accumulation of a sufficient body of data on chemically defined substances to make clear the broad outlines of the relationship between chemical structure and immunological reactivity. Indeed, the application of immunochemical methods has extended far beyond the study of immunity to disease and has become a valuable tool in the characterization of proteins and polysaccharides.

For those not familiar with the terminology of the immunologist, a short resumé of the basic concepts and nomenclature is given.

When an animal receives one or more parenteral injections of certain foreign materials—proteins, red blood cells or tissue extracts from another species, or of bacteria or bacterial products—there generally appear in the serum, within a few days, substances which possess the unique property of reacting with the material injected. These substances are termed *antibodies* and the materials which stimulated their production are called *antigens*. An animal which has formed antibodies after receiving injections of an antigen is said (by convention) to be immunized. The presence of antibodies may be recognized by the occurrence of certain characteristic reactions when a serum containing antibody is mixed with its antigen. For example, when the serum of an animal which has been immunized with red blood cells of another species is added to a suspension of the erythrocytes, clumping of the cells is observed. This phenomenon is called *agglutination* (I, 3), and the antibody in the serum which combines with the red cells to cause it is known as an *agglutinin*.

If a soluble antigen such as crystalline egg albumin is mixed in suitable proportions with its immune serum a precipitate consisting

of antigen and antibody forms. This is termed a *precipitin reaction* (I, 2) and the antibody is spoken of as a *precipitin*.

When fresh antiserum prepared by injecting certain bacteria or erythrocytes is added to a suspension of the antigen, dissolution or *lysis* of the bacteria or red blood cells is observed. Antibodies producing this type of reaction are called *lysins*, and depending upon whether their action is directed toward bacteria or red blood cells, they are termed *bacteriolysins* or *hemolysins* respectively. For hemolysis or bacteriolysis to occur, the immune serum used must be fresh. If old serum is used, or if the serum has been heated to 56° C. for 30 minutes before it is added to the suspension of cells, lysis does not occur, but agglutination is frequently observed. The lytic action of the immune serum may be restored by addition of fresh normal serum from a variety of animals, notably the guinea pig. This thermolabile factor in normal serum necessary for lysis is called *complement* (I, 4) or *alexin*, and the reaction of antigen and antibody with complement is called *complement fixation* (I, 4).

Other manifestations of the presence of antibodies can be observed *in vivo*. Certain bacteria, notably the organisms causing diphtheria and tetanus, produce soluble toxic proteins. These *toxins*, depending on the amounts administered, produce a variety of localized lesions and systemic reactions which may be fatal. Toxins, however, are antigenic and if animals receive initial small sublethal doses of toxin or of toxin detoxified by formalin or other means (*toxoid*) they soon acquire a tolerance or capacity to withstand many times the amount of toxin which would be lethal on first injection. Such animals are said to be immune to the effects of the toxin and their serum contains *antitoxins* which if mixed with a given amount of toxin, partially or totally inhibit or neutralize the toxic effects of the latter. These antitoxins, if injected into the skin, also prevent the development of a localized reaction when toxin is injected into the same site.

If virulent bacteria are injected into an animal which has previously received a sufficient amount of an appropriate immune serum, the animals may survive infections which would otherwise be fatal. The antibodies responsible are referred to as *protective antibodies*. Protective antibodies may be formed in response to infection in which case recovery is determined by the capacity of the animal to form such antibodies. Indeed, the science of immun-

ology grew out of the observation that recovery from many infectious diseases was accompanied by a greatly decreased susceptibility to reinfection or was followed by complete resistance. Attempts to produce immunity dating back to antiquity were based on the artificial inoculation of infected materials from mild cases into healthy individuals.

Another *in vivo* manifestation of the presence of antibody is the production of *anaphylactic shock* (I, 5). It occurs when an animal such as a guinea pig receives a second injection of an antigen like crystalline egg albumin ten to twelve days or more following an initial injection. If the second injection is given intravenously, an immediate reaction characterized by chewing, coughing, dyspnea, convulsions and death due to suffocation may result. Anaphylaxis may also be produced by injection of guinea pigs with a serum containing antibody followed by injection of the corresponding antigen one or two days later. Animals which received an initial injection of antigen are said to be *actively sensitized* and those which are given an initial injection of serum containing antibody are termed *passively sensitized*. The ten to twelve day interval between the sensitizing and shocking injection in actively sensitized animals is necessary for antibodies to be formed to the antigen. These may then combine with the second or shocking dose of antigen to produce anaphylaxis.

Antibodies may show several other activities. If they promote phagocytosis of their homologous antigens, they are called *opsonins*. Certain antibodies may sensitize local areas of skin so that an inflammatory type of response occurs when antigen is injected into the same site.

These various immune reactions are often merely different manifestations of activity by the same substance—antibody. For example, a suitable amount of antibody to the type-specific capsular polysaccharide of the pneumococcus, agglutinates suspensions of pneumococci of the same type, precipitates or fixes complement, with the purified homologous type-specific polysaccharide, sensitizes guinea pigs passively, and protects mice against infection. It has been shown that all of these reactions are due to one and the same antibody. The type of reaction depends on the manner in which the tests are carried out, whether with particulate or soluble antigens, or in the presence of complement, etc.

When a mixture of antigens is injected, antibodies specific for each antigen are formed. When one speaks of *an antibody*, one means the antibody formed against a *single* antigen. Antibodies should, therefore, be designated in terms of their specificity for their corresponding antigen, e.g. anti-egg albumin, rather than in terms of the way their activity is manifested. The various antibodies formed in response to a mixture of antigens are distinct and can be removed separately from the serum by addition of each antigen singly. If a suitable quantity of a soluble or particulate antigen is added to immune serum, all of the antibody to this antigen is removed in the precipitate or agglutinate, and the supernatant is freed of this antibody. Removal of antibody from an antiserum by antigen is called *absorption*, and is frequently used to obtain antisera specific for a given single antigen, when the antigen is not readily available in pure form. For example, a type I anti-pneumococcal rabbit serum contains antibodies to the type I specific carbohydrate, to a group-specific carbohydrate ("C" substance) and to one or more proteins of the pneumococcus. Since the group-specific carbohydrate and protein appear to be present in pneumococci of other types, absorption of the type I serum with type II pneumococci, or with an R (rough) (Dawson S) strain from type I, which does not contain the type I polysaccharide (I, 3) will remove these antibodies leaving antibody only to the type I polysaccharide.

Quantitative studies have indicated that even antibodies to a single antigen, such as crystalline egg albumin are not uniform, but individual antibody molecules vary in the nature and number of their reactive groups (I, 2, 6; II, 9). They may still be considered to behave as a single substance however, when comparisons are made between a number of totally unrelated antigens, since all of the various antibody molecules to each antigen can react specifically with their own antigen.

CHEMICAL NATURE OF ANTIBODIES

Antibodies are now generally accepted as proteins and belong to the class of serum globulins. Doubts of earlier investigators as to the protein nature of antibodies were finally resolved by the accumulation of an overwhelming mass of evidence culminating in the preparation of analytically pure antibodies and the deter-

mination of their molecular weights and other physico-chemical properties (I, 6). (For earlier work see (I).)

As yet little or nothing is known of the specific modifications of chemical structure required for the formation of an antibody molecule instead of a molecule of normal serum globulin. In most instances, these changes must be very subtle since no method of distinguishing between certain normal globulins and antibodies has been found. Even as antigens, normal and antibody globulins formed in the rabbit have been found to be identical (2).

The unique property of *specificity* shown by antibodies—that is, their capacity to react only with the substance used to produce them or with substances showing a very close chemical relationship to the homologous antigen (cross-reactions) (II, 9) (3)—has been responsible in large measure for their tremendous importance to the chemist, since it has made possible not only the detection but also the quantitative estimation of antigens in mixtures which could not otherwise be resolved (4). Immunological specificity has also provided a basis for classifying animal species that correlates closely with classifications established from morphological and paleontological considerations (5). The immunological classification of animal species is based on the observation that cross-reactions occur among antigens of closely related species, the degree of cross-reaction decreasing and disappearing as the relationship becomes more remote.

CHEMICAL NATURE OF ANTIGENS

The large majority of antigenic substances are protein in nature, and until recently it was generally believed that materials other than protein could not function as antigens, i.e. stimulate production of antibodies unless in chemical combination with protein. This concept became untenable when it was clearly demonstrated that injection of purified type-specific capsular carbohydrates of pneumococci induced active immunity in mice (6-9) and in man (10-13) although these polysaccharides were not antigenic in the rabbit (8), findings which for the first time established that antigenicity was to some extent a function of the species immunized.

With regard to the specificity of protein or tissue antigens, it has long been known that if animals were immunized with such an antigen from a given species, the antibodies formed would react

most intensely with the homologous antigen but would react to a lesser degree or not at all with similar antigens from other unrelated species. This phenomenon was termed *species specificity*. Certain exceptions were noted, however, in which cross-reactions occurred with antigens prepared from a given organ such as kidney, thyroid, crystalline lens of a large number of unrelated species. This cross-reactivity is known as *organ specificity* and appears to be due to similarities in chemical structure of such antigens (II, 9). With purified materials from tissues or organs, three types of specificity may be observed: 1. Serological properties common to different tissues of the same species. 2. Specificity common to the same tissue or organ of different species. 3. Specificity characteristic only of one organ of a given species (14). In other instances, certain antigens termed *heterogenetic* appear to be widely distributed in nature without regard to degree of relationship (3).

When cross-reactions occur between closely related antigenic substances, it can be demonstrated that the cross-reacting antigen generally does not remove all of the antibody to the homologous antigen. With systems giving precipitin or agglutination reactions, this is usually accomplished by addition of successive portions of the cross-reacting antigen and removing the precipitated or agglutinated antigen-antibody complex formed after each addition by centrifugation until visible precipitation or agglutination no longer occurs. This procedure is known as absorption of cross-reacting antibody. After these antibodies have been absorbed, the supernatant serum may be tested with homologous antigen for the remaining antibody which does not cross react.

METHODS OF MEASURING THE QUANTITY OF ANTIBODY

Numerous methods have been devised for estimating the amounts of antibody in a given antiserum. These may be classified into two general types—*in vivo* or *in vitro* methods. The former make use of experimental animals while the latter are based on the visible reactions which occur when antigen and antibody combine.

In vivo methods of estimating the antibody content of an antiserum measure the relative capacity of an antiserum to protect experimental animals against standardized lethal doses of toxins or of virulent bacteria, or may determine ability to neutralize local manifestations of the antigen such as the cutaneous reactions

produced by toxins, or to produce local or generalized passive sensitization.

One of the most widely used of these *in vivo* tests is the mouse protection test. In the standardization of antipneumococcal serum, one mouse protective unit has been arbitrarily defined as that fraction of a cubic centimeter of serum which will protect a given proportion of mice against one million fatal doses of an 18-hour serum-broth culture of such virulence that 3 to 10 organisms will produce death in 36-48 hours when injected intraperitoneally (15).

A typical procedure is as follows (16): Mice are injected intraperitoneally with 0.5 ml. of 1:200 dilution of an 18-hour broth culture of pneumococcus (500,000 lethal doses) mixed with an equal volume of the dilution of serum to be tested. Dilutions of serum of 1:25, 1:50, 1:100, 1:200, 1:400, 1:600, 1:800, 1:1000, 1:1200, 1:1600, and 1:2000 of serum are used and three mice are injected with each dilution. The highest dilution of serum protecting 2 of 3 mice for 96 hours is taken as the number of protective units.

The technic employed in protection tests with bacteria varies widely and the definition of a mouse protection unit may be different for each organism. To obtain maximum virulence with certain organisms such as meningococci (17), typhoid and dysentery bacilli (18), the bacteria are suspended in mucin (Wilson 1701W). Many investigators inject the serum one-half or one hour before the infecting dose (19, 20), instead of mixing the two. Rake (19) reports that antimeningococcal serum gives the same degree of protection whether inoculated 1 hour before or 1 hour after the culture.

Antitoxic potency of sera is measured by comparison with a standard sample of antitoxin. This procedure is necessary since the potency of crude toxins is found to vary and solutions may contain toxin and toxoid in indeterminate amounts, both of which combine when mixed with antitoxin. In carrying out the test, increasing amounts of toxin are added to a series of tubes containing one unit of the standard antitoxin and each of the mixtures is injected into a 250 gram guinea pig. The amount of toxin which must be mixed with one unit of antitoxin to cause death in 4 days is taken as the end-point and is called the L+ dose; the amount which can just be neutralized by one unit of antitoxin is called the L₀. To determine the potency of the unknown serum, increasing dilutions of the

antitoxin are each mixed with one L+ dose of toxin and the mixtures injected into guinea pigs. The volume of serum which when mixed with one L+ dose of toxin results in death in four days contains one unit of antitoxin. End-points using the L₀ dose are less precise, since estimation of the point at which complete neutralization occurs is more subjective.

Protection and neutralization tests are subject to considerable experimental error as are all types of assay requiring animals. If a sufficient number of animals is used for each dilution and the successive decrements of serum are sufficiently small, the error can be maintained within ± 10 to 20 per cent.

It must be recognized that protection and neutralization tests constitute the only reliable primary reference standard for the therapeutic potency of any serum, and before any *in vitro* method of measuring antibody content can be used as an index of therapeutic potency, it must first be demonstrated that it yields results correlating closely with protective power as measured by the procedures outlined above. With complex mixtures of antigens such as bacteria, only one or two of the antigens present may give rise to protective antibodies, while many others may give rise to agglutinins, precipitins, etc. and unless the purified antigens responsible for the protective potency are available for *in vitro* tests, it is unlikely that any correlation between protective potency and total antibody content would be found. As will become apparent later, one of the main efforts of immunochemistry has been to separate the antigens responsible for protection and to use them for immunization in man. This has as yet been accomplished in only relatively few instances (cf. 20a).

The *in vitro* methods of measuring antibody potency fall into several general types regardless of which of the immune reactions is used:

1. Dilution Methods: The classical methods of comparing immune sera use as the end-point the highest dilution of serum at which a detectable reaction, such as precipitation, agglutination, lysis, or complement-fixation occurs, when added to a constant amount of antigen. The serum dilution giving this end-point is known as the *titer*, and a serum which agglutinates at a dilution of 1:25,000 is considered five times as potent as one which agglutinates at a dilution of 1:5,000. Usually the dilution of serum is doubled in

successive tubes and if the same test is repeated several times, it will soon become evident that the end-point may be in error by a factor of two. Dilution methods are widely used in diagnosis and provide a rapid means of obtaining comparative data on the potency of various antisera.

2. Optimal Proportions Methods: These procedures determine the ratio of antigen and serum at which floccules appear most rapidly. They are based upon the assumption that the rate of flocculation is a measure of the combination of antigen and antibody and will occur most rapidly when the relative proportions are most favorable. The technic introduced for the assay of precipitating sera by Dean and Webb (21) consists in mixing varying dilutions of antigen with a constant volume of serum in a series of tubes, the total volume in each tube being constant. The tube in which flocculation first occurs is noted, and the ratio of antigen dilution to serum dilution in this tube is calculated. For example, if the serum was used undiluted and flocculation occurred most rapidly when added to a 1:10 dilution of antigen, the optimal ratio would be 1:10—one part of antigen combines with 10 parts of serum. Another serum with an optimal ratio of 1:50 would be only one-fifth as potent. The optimal proportions method is more precise than the dilution method, but also enables only comparisons of relative antibody content to be made.

Ramon (22) had previously developed the optimal proportions principle for the assay of diphtheria antitoxin. The reverse procedure is used however in that increasing dilutions of antitoxin are added to a constant amount of toxin, and the ratio of the dilution of antitoxin to toxin giving most rapid flocculation is taken as the end-point. This is called the constant antigen optimal ratio in contrast to the Dean and Webb constant antibody optimal ratio. These two ratios are not the same (23) and neither optimum appears to correspond necessarily to the point of complete removal of antibody (23-26). In some instances most rapid flocculation occurs in the region of antibody excess (25), whereas in others it occurs when antigen is in excess (26). Results of Ramon titrations are expressed in Lf units; one Lf unit represents the amount of toxin giving most rapid flocculation with one standard unit of antitoxin.

3. Quantitative Chemical Methods: This group of methods, which has come into widespread use, permits measurement of

amounts of antibody on a weight basis with a precision conforming to the requirements of analytical chemistry and has contributed materially to making immunochemistry an exact science. The bulk of this volume will be concerned with the technics, applications and interpretation of results obtained with these procedures.

These quantitative chemical methods take advantage of the specificity of immunological reactions and of the fact that antibodies are proteins. Since on addition of antigen only antibody is removed from the serum, analysis for nitrogen can provide a measure of the amount of antibody. To measure the antibody content of a serum, an amount of a soluble or particulate antigen is added sufficient to remove all of the antibody as an insoluble precipitate or agglutinate. This usually occurs when a slight excess of antigen is present. The precipitate or the agglutinated bacteria are centrifuged off, washed free from serum protein and analyzed for nitrogen by the micro Kjeldahl or any other suitable method. Antibody nitrogen is calculated by subtracting the nitrogen of the added antigen from the total nitrogen found.

METHODS OF MEASURING AMOUNTS OF ANTIGEN

The three general methods outlined above can all be adapted to the measurement of quantities of antigen. Using the dilution or optimal proportions method, a given serum is standardized by adding increasing known amounts of antigen to a constant volume of serum and determining the minimum quantity giving visible precipitation or the ratio giving optimal flocculation. Unknown solutions may then be analyzed in a similar manner and the ratios of the dilution of the known to the unknown solution at the end-point gives the concentration of the unknown solution relative to the standard. For accurate results, comparison of the known and standard should be made simultaneously.

Use of the quantitative methods for the estimation of antigens and also of complement will be considered subsequently.

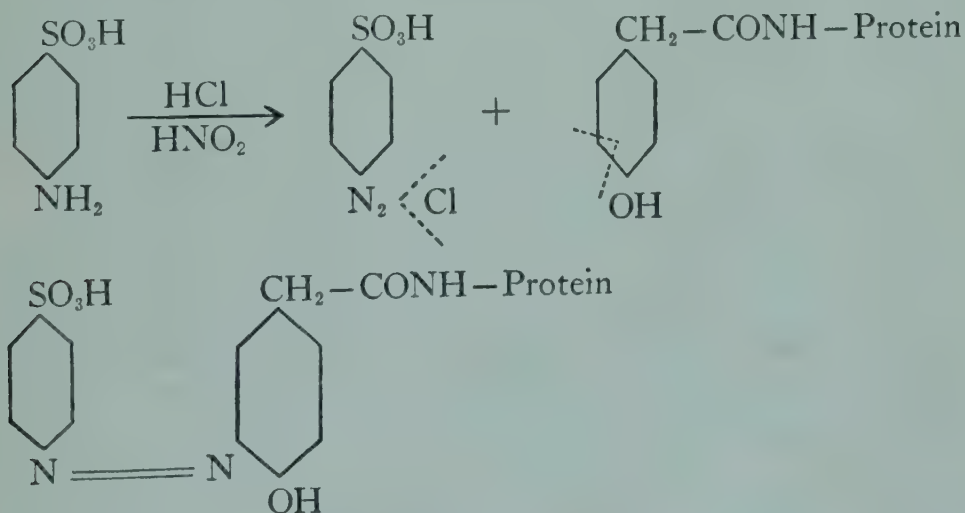
CHEMICALLY ALTERED ANTIGENS

Since little is known about intimate details of protein structure, such as the order and arrangement of amino acid residues in the molecule, the differences in the immunological specificity revealed by the study of native proteins could not readily be explained except

in very general terms, and it was necessary to use other methods involving the introduction of known chemical groups into proteins to establish the chemical basis for the specificity of serological reactions. With the discovery of specific polysaccharides composed of only a few component sugars, it became possible to correlate specificity with chemical structure in some naturally occurring systems (II, 9).

Much of our knowledge of the role of chemical structure in determining immunological specificity, however, resulted from studies on the effects of introducing chemical substituents of known structure into proteins (II, 10). Treatment of proteins with iodine or nitric acid was found by Obermayer and Pick (27) to alter the properties of proteins so that their species specificity was greatly reduced and to result in the appearance of a new specificity characteristic of the introduced nitro or iodo group as manifested by a capacity to react with nitro or iodo compounds prepared with proteins of unrelated species.

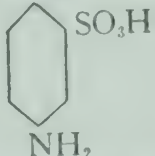
The most extensively used method of introducing new substituents into proteins consists in formation of azo proteins by coupling with diazo compounds of the desired chemical structure (28). For example, sulfanilic acid may be diazotized and coupled mainly to the tyrosine groups of a protein such as crystalline egg albumin by the following reactions:



If rabbits are immunized with such an azo protein, the antibodies formed may cross-react with sulfanilic acid coupled to an unrelated protein, and in general will react only slightly with egg albumin. A

guinea pig sensitized with one sulfanilic acid azo protein will respond with a typical anaphylactic reaction when sulfanilic acid coupled to another protein is injected (1). By adding a solution of sodium sulfanilate to the serum, the precipitation by sulfanilic acid azo protein can be inhibited. These findings indicate that the specificity is in large measure determined by the introduced group (3).

Using antisera to such azo proteins, the effects of changes in the chemical structure of the introduced grouping on the specificity can be evaluated in several ways. If an azo protein in which the sulfonic acid group is in the meta position is prepared, from m-amino

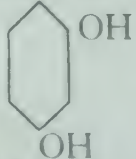
benzene sulfonic  the degree of reaction with an anti-

serum to the sulfanilic acid azo protein can be studied. In addition, the relative effectiveness of sulfanilic acid and meta-amino benzene sulfonic acid in inhibiting precipitation by the azo proteins can also be compared.

The large mass of accumulated data on the effects of such chemically introduced determinant groups has indicated that cross reactions are a function of similarity in chemical structure and spatial configuration. For details of such studies see (1, 3, 34). Methods of introducing a variety of substituents into proteins are considered in Section IV. Most of the studies on such chemically altered proteins were carried out by coupling to complex mixtures of proteins such as horse serum. This made it impossible to apply quantitative chemical methods in studying such systems.

In the instances in which azo proteins were prepared using individual proteins, evidence has been obtained indicating that different results may be obtained when the same substituent group is introduced into several proteins (29, 30, 31) and that all such azo proteins do not necessarily cross-react (II, 10).

By coupling certain of these introduced chemical groups to sub-

stances like resorcinol,  compounds may be obtained in

which more than one substituent group is present in a molecule.

In some instances, these simple substances precipitate with antisera to an azo protein containing the same groups. For example, Landsteiner et al. (32) have found that p-amino succinanilic acid, $\text{H}_2\text{N}-\text{C}_6\text{H}_4-\text{NH}-\text{CO}-\text{CH}_2-\text{CH}_2-\text{COOH}$, coupled to resorcinol so that two succinanilic acid groups were linked to each resorcinol molecule would precipitate with an antiserum prepared from p-amino-succinanilic acid coupled to protein. Similar studies have been carried out with a large number of other substances (33, 34).

Substances which on injection do not give rise to antibodies, but which are able to react with antibodies to produce either precipitation or to inhibit precipitation have been termed *haptens* (1, 3). This definition has been used to include not only the simple chemical substances which are determinants of specificity when conjugated to protein, and which inhibit precipitation (1, 3, 33, 34), but also substances obtained from natural sources such as the pneumococcal type-specific polysaccharides which are not antigenic in the rabbit (1, 3) (35). More recent studies, however, have established that they function as complete antigens in other species, such as the mouse and man, unless degraded in the course of purification. In this volume, the use of the term *hapten* will be confined exclusively to non-antigenic groups artificially introduced into proteins which affect specificity. Use of the term in this sense serves to exclude the possibility of confusion resulting from differences in the response of various species to immunization or from degradative changes in the course of isolation.

A number of substances, notably those of the Wassermann and Forssman type, obtained by alcoholic extraction of beef heart or of horse kidney, respectively, and from a wide variety of other sources, do not stimulate antibody production on injection. However, if administered as a mixture with serum of a foreign species (pig serum is generally used) or in some cases if adsorbed on kaolin or aluminum hydroxide (36), complement-fixing antibodies may be produced (3, 36). Similar results have been reported with a number of phosphatides such as lecithin, and sterols like cholesterol, hydroxycholesterol and dehydrocholesterol (for discussion and references see 3). As yet little is known about the chemical basis for the action of foreign serum in rendering such substances antigenic (3, 36).

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CHAPTER 2

PRECIPITIN REACTION

The discovery and naming of the precipitin reaction is attributed to Kraus (1) who observed that a precipitate formed when cell-free filtrates of broth cultures of the typhoid bacillus were mixed with anti-typhoid serum. It has since been generally recognized that precipitating antibodies (precipitins) can be produced against most proteins and also against some polysaccharides.

While there are numerous modern theories of the mechanism of the precipitin reaction (cf. Heidelberger, Marrack, Boyd, Hershey, Kendall, Pauling ref. 11, 26, 21, 22, 23, 25, 24) it is generally accepted that the specific precipitate is formed as the result of union between antigen and antibody molecules, followed by separation from solution of the insoluble antigen-antibody complex.

The difference between specific precipitation and agglutination arises chiefly from the disparity in size of antigen particles. When the antigen has molecular dimensions, that is, when it is in solution, it is appropriate to describe the clumping by antibody as precipitation. In the case of antigen particles large enough to be sedimented by centrifugation at about 1000-2000 r.p.m., or large enough to appear as discreet particles under the microscope, the aggregation of the suspended particles by antibody is properly described as agglutination. Both agglutinin and precipitin reactions are alike in that they result from the specific union of antigen and antibody.

The high degree of specificity and the great sensitivity of serological reactions make it possible to distinguish between and to identify different proteins or polysaccharides by means of precipitin tests. Nuttall (4) tested blood sera from numerous animal species with immune rabbit sera and showed the reaction to be strongest with the kind of serum used for immunization while with other sera the intensity of reaction was found to parallel the degree of zoological relationship. Heidelberger and Landsteiner (3) found that a rabbit antiserum to crystalline horse hemoglobin gave a cross reaction only with donkey hemoglobin. A variety of other hemoglobins from less closely related species were inactive or only slightly active toward this serum. Hektoen (5) showed that

the thyroglobulins of different species are closely related serologically (organ-specificity) but are not necessarily identical. Stokinger and Heidelberger (6) demonstrated by quantitative methods the precise extent of the serological relationship in a number of rabbit antisera between thyroglobulins of several species. Beef, sheep and hog thyroglobulin were found to cross-react extensively but human thyroglobulin differed markedly giving less than 20 per cent cross reaction (cf. II, 9).

Of great practical importance is the forensic use of precipitin tests in differentiating human and animal blood or in the detection of adulteration of foodstuffs.

Impurities present even in minute amounts may readily be detected in partially or even in highly purified biological substances. For example, Goldsworthy and Rudd (7) were able to demonstrate globulin in repeatedly recrystallized horse serum albumin. Preparations of type-specific pneumococcus polysaccharide frequently contain the group specific "C" substance as shown by precipitin tests with antisera to pneumococci of a type different from that of the polysaccharide to be tested. As pointed out by Kabat (8), precipitin tests should be useful to detect normal tissue antigens in purified virus preparations (II, 8). Apart from the specificity of serological reactions, their great sensitivity renders them eminently suited to the detection of traces of antigenic materials. Using potent antisera, it is possible to detect as little as 1 microgram of egg albumin or 0.1 microgram of pneumococcus type-specific polysaccharide in 1 ml. of fluid. With the quantitative precipitin method, it is possible to measure small amounts of antigen with a degree of accuracy as great as that of many standard chemical analyses.

While there are numerous ways of describing the intensity of precipitin reactions, only those based on the determination of the amount of specific precipitate either by direct weighing (8a) or by chemical analysis (9-13) yield absolute results and meet the requirements of quantitative analytical chemistry. Other technics, like the serum dilution titer, the optimal proportions method, turbidity measurements, determination of the volume of specific precipitate, the optimal flocculation method, all give results in relative terms rather than in absolute units (cf. exptl. procedures) and are much less precise. While these methods are often useful when a quantitative answer is not required, the discussion of the precipitin reaction

and its characteristics will be given mainly in quantitative terms.

Analyses of washed specific precipitates were first carried out by Wu (9, 10) who attempted to calculate their composition with respect to antigen and antibody.

The quantitative precipitin technique as an analytical method for the estimation of antibody was elaborated and perfected by Heidelberger and Kendall (11, 12, 13), and has been widely adopted as a reliable and strictly quantitative method. It is based on analysis of the washed specific precipitate for N by the micro Kjeldahl method after complete precipitation of antibody by addition of antigen in slight excess. Since specific precipitates are composed of antibody as well as antigen, the analysis for N will not represent antibody N unless the antigen is a nitrogen-free substance. In the case of protein antigens, it is necessary to subtract the antigen N added (if not in large excess) from specific precipitate N in order to obtain antibody N.

As usually carried out, quantitative precipitin determinations employ samples from 0.10 to 1.0 mg. of antibody N; a micro modification for samples as low as 0.01 to 0.02 mg. N has also been developed (32) and this has been useful for measuring the amounts of antibody in human sera after immunization or during convalescence from various infections (34).

Although the quantitative precipitin estimation is simple to perform, its critical evaluation requires familiarity with certain general principles. These may be illustrated by detailed description in quantitative terms of a typical case, the egg albumin-anti-egg albumin (Ea-anti Ea) system (13). Although there may be differences with respect to some particular feature, the general principles have been found to apply to a variety of precipitin systems.

Table 1 and fig. 1 show the type of data obtained in the egg albumin system when increasing amounts of antigen are added to a series of tubes containing a constant volume of antiserum. The contents are mixed and the tubes are placed in the icebox. After 48 hours they are centrifuged in a refrigerated centrifuge. The precipitates are washed twice with saline in the cold and analyzed for N by the micro Kjeldahl method. For convenience, results are expressed in terms of nitrogen rather than protein. Determinations are usually carried out in duplicate and the figures in the table represent averages. Portions of each supernatant are tested by

TABLE I

Addition of Increasing Amounts of Egg Albumin to 1.0 Ml. Serum 3.87, 1:1, at 0°

Ea N added	Ea N pptd.	Total N pptd.	Anti-body N by difference	Ratio anti-body N:Ea N in ppt.	Anti-body N pptd., calcd. from eq. [4]	Anti-body N pptd., calcd. from eq. [5]	Tests on supernatant
mg.	mg.	mg.	mg.	.	mg.	mg.	
Course I							
Mg. antibody N pptd. = $15.8 \text{ Ea N} - 83 (\text{Ea N})^2$							
Mg. antibody N pptd. = $19.4 \text{ Ea N} - 36 (\text{Ea N})^{3/2}$							
0.009 ¹	Total	0.156	0.147	16.2	0.137	0.146	Excess A
0.015 ⁵	Total	0.236	0.220	14.2	0.225	0.231	Excess A
0.025	Total	0.374	0.349	14.0	0.343	0.342	Excess A
0.040	Total	0.526	0.486	12.2	0.499	0.488	Excess A
0.050	Total	0.632	0.582	11.6	0.582	0.567	Excess A
0.065	Total	0.740	0.675	10.4	0.677	0.664	Excess A, trace Ea
0.074	Total	0.794	0.720	9.7	0.714	0.710	No A or Ea
0.082	Total	0.830	0.748	9.1	0.738	0.746	No A, < 0.001 Ea N
0.090	0.087	0.826	0.739	8.5	0.746	0.763	Excess Ea,
0.098	0.089	0.820	0.731	8.2			Excess Ea,
0.124	0.087	0.730	0.643	7.4			Excess Ea,
0.135	(0.072)	0.610	(0.538)	(7.5)			Excess Ea,
0.195	(0.048)	0.414	(0.366)	(7.6)			Excess Ea,
0.307	(0.004)	0.106					Excess Ea,
0.490		0.042					

Maximum Ea N, A N in ppt. according to equation [4] 0.095, 0.752; according to equation [5] 0.129, 0.836

Values in parentheses are considered uncertain

From (13)

addition of antigen and antiserum to determine whether excess of antibody or antigen is present. The first and third columns in the table show the amount of antigen nitrogen added and the total (antigen + antibody) nitrogen precipitated (fig. 1., curve IV). It is seen that for the serum used curve IV rises swiftly to a maximum of 0.83 mg. N at about 0.09 mg. Ea N. Addition of more than this optimal amount of Ea yields progressively less precipitate N, due to formation of soluble compounds.

Inspection of the supernatant tests (table I, last column) shows the existence of three zones, i.e., a region of antibody excess, a narrow equivalence zone where neither antigen nor antibody is found in the supernatant, and finally a region of antigen excess. At the beginning of the antigen excess region all of the antibody and all but a very small part of the antigen are precipitated, but when antigen is present in considerable excess soluble compounds are formed which remain in the supernatant together with uncombined antigen so that precipitation is less than maximal. This

inhibition of precipitation may become complete in some antigen-antibody systems with a large excess of antigen.

The Ea-anti Ea system is extremely sensitive to inhibition by excess antigen so that even a moderate excess of antigen yields less than the maximum precipitable N. Other reactions, for example those of pneumococcus polysaccharide with rabbit and horse anti-

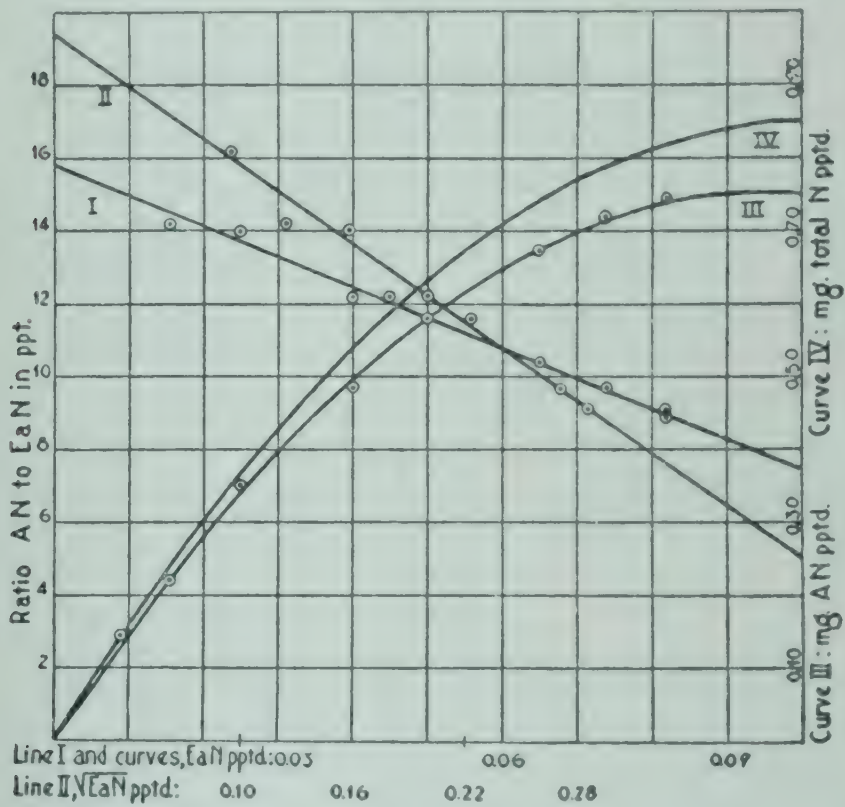


FIG. 1. Quantitative course of the precipitin reaction between egg albumin and rabbit anti-egg albumin. From (13).

serum, are less readily inhibited, i.e., require a greater excess of antigen for inhibition. (See below for a more detailed discussion of inhibition). If tests on the supernatant show that no antigen is present (i.e. in the region of antibody excess), all the antigen is considered to be in the precipitate (provided the antigen is a single substance) and antibody N (column IV, table 1) is calculated by subtracting the antigen N added from the total N precipitated (fig. 1, curve III). If the antigen is a polysaccharide free from nitrogen no subtraction is, of course, necessary. (The evidence for complete precipitation of antigen in the antibody excess zone which

justifies this calculation is discussed below) (cf. ref. 6, 13, 14). Dividing the antibody N precipitated by the amount of antigen N added gives the ratio Ab N/Ea N in the precipitate, the values of which are given in table 1, column 5. In the region of appreciable antigen excess, determination of the amount of Ea N in the precipitate is carried out by analysis of the supernatant for Ea as described in detail below, and subtraction of the value found from the quantity of Ea N added.

Inspection of the precipitin curves and of the Ab N/Ea N ratios shows that the composition of the precipitate varies continuously with changes in the proportion of the reactants. In the present case, i.e., the Ea system, the aggregates formed at the extreme end of the antibody excess zone have a nitrogen weight ratio of Ab/Ea of about 20, corresponding to a molecular composition of Ab_5Ea . In the equivalence zone the molecular composition is about Ab_3Ea to Ab_5Ea_2 . When the reaction is partly inhibited by excess antigen, the composition becomes about Ab_2Ea . The soluble compounds in the inhibition zone might then consist of Ea and Ab in equimolecular proportions, i.e. $AbEa$ (cf. 26). Table 2 summarizes some of the data on the molecular composition of specific immune precipitates from rabbit sera.

TABLE 2
Molecular Composition of Specific Precipitates from Rabbit Antisera

Antigen	Molecular Weight	Molecular ratio of antibody to antigen		
		Extreme antibody excess zone	Equivalence zone	
			antibody excess side	antigen excess side
Egg albumin.....	42,000	5	3	2.5
Horse serum albumin.....	67,000	6	4	3
Thyroglobulin.....	700,000	40	14	10
Viviparus hemocyanin.....	6,630,000	...	120	83
Tobacco mosaic virus.....	33,000,000	900	...	450

Modified from (27)

When the ratio Ab N/Ea N in the antibody excess zone is plotted against the antigen N added, a straight line is obtained (fig. 1, line I), which follows the general type of equation

$$\frac{\text{Ab N pptd.}}{x} = a - bx \quad [1]$$

where x is the amount of antigen N added. The constants a and b in this empirical relation may be evaluated from line I (fig. 1): a equals the intercept on the y axis and b equals the slope of the line. For the data in table 1 (fig. 1, line I), the equation becomes

$$\frac{\text{Ab } N \text{ pptd.}}{x} = 15.8 - 83x \quad [2]$$

Multiplying both sides of equation [1] by x gives the equation

$$\text{Ab } N \text{ pptd.} = ax - bx^2 \quad [3]$$

and substituting $a = 15.8$ and $b = 83$ the equation which describes curve III, fig. 1, becomes:

$$\text{Ab } N \text{ pptd.} = 15.8x - 83x^2 \quad [4]$$

Equation [4] expresses the relation between the amount of Ab N precipitated and the amount of antigen N added throughout the region of antibody excess up to the point of maximum precipitation. Equations of the same general form have been found applicable to the various precipitin systems which have been studied quantitatively. Different antisera in any given system will yield equations in which the constant a depends on the characteristics of the antibody, and b depends both on the characteristics of the antibody and the amount present.

When it is desired to compare the combining characteristics of the antibody in different immune sera it is convenient to eliminate the factor of antibody content from the equation by reducing the data obtained on sera of different antibody content to a common denominator of 1 mg. total antibody N per ml. This is done by dividing all values for antibody precipitated and antigen added by the amount of maximum precipitable antibody N . Equations on such adjusted data permit comparisons to be made between the characteristics of antibody in different sera (15, 16) or between purified antibodies and antibody fractions and their original sera (17). In this connection, it should be mentioned that the antibody in an immune serum does not possess uniform characteristics. If part of the antibody is removed from a serum by specific precipitation, the

remainder in the supernatant will generally not yield the same equation as the original serum (13, 19).

Another empirical equation of the form

$$\text{Ab N pptd.} = cx - dx^{3/2} \quad [5]$$

has been found to fit the data in certain systems (13, 18, 19) better than the quadratic equation [4]. The constants c and d are evaluated by plotting $\text{Ab N}/\text{Ea N}$ against $\sqrt{\text{Ea N}}$ (line II, fig. 1). The intercept on the y-axis gives $c = 19.4$ while the slope of the line yields $d = 36$, giving the equation,

$$\text{Ab N pptd.} = 19.4 \text{ Ea N} - 36 (\text{Ea N})^{3/2},$$

in table 1.

Heidelberger and Kendall (20) derived a theoretical equation, also of quadratic form, which is based on the mass law of chemical reactions.

$$\text{Ab N pptd.} = 2 Rx - \frac{R^2}{A} x^2 \quad [6]$$

The constant A equals the number of milligrams of antibody N precipitated at the equivalence point (mid-point of the equivalence zone) and R represents the ratio of A to milligrams of antigen N precipitated (added) at the same reference point.

Equation [6] was derived on the assumption of a series of competing bimolecular reactions between antigen and antibody (20). This treatment has been criticized by Marrack (21), Boyd (22), and Hershey (23) as well as by Pauling who, however, obtained an equation of similar form starting with other assumptions (24). An alternative derivation by Kendall (25) meets some of the objections to the earlier development (20).

The theoretical significance of a and b in the empirical equation [3] is now clear, for comparison with equation [6] shows that $a = 2R$ and $b = \frac{R^2}{A}$. In the empirical equation [5] the constant $c = 3R$ and $d = 2\sqrt{\frac{R}{A}}$ (18).

The mathematical treatment leading to equation [6] involves only the formation of compounds having ratios between R and $2R$

but experimental data show that in certain systems compounds of ratio larger than 2R are encountered. By extending the process of calculation, Heidelberger and Kendall (20) derived the equation:

$$\text{Ab N pptd.} = \frac{A - 2(A - R_x)^4}{A(A - R_x)^2 + A^2}$$

[7]

which applies to systems in which the ratio varies between R and 3R. As pointed out by Heidelberger (26) it is usually possible to avoid the complicated equation [7] and use the simpler equation [6] by choosing the reference point for R and A at either end of the equivalence zone, depending on the system studied, instead of at the center of the equivalence zone. The difficulty of choosing the reference point is best avoided by using the empirical equation [1] which permits an unambiguous evaluation of the necessary constants.

To illustrate the applicability of equations [3] [5] and [6] to various precipitin systems, data on several cases are given in tables 3, 4, 5, 6, representing, respectively, the horse (20) and rabbit (16) anti-pneumococcus type-specific polysaccharide system, and two widely different protein-anti-protein systems, namely horse serum albumin (19) and thyroglobulin (6) and their homologous rabbit antibodies.

TABLE 3
Addition of Increasing Amounts of S III to Horse Antipneumococcal Sera and Antibody Solutions and Comparison of Experimental Data with Values Calculated According to:
 $N \text{ Precipitated} = 2 RS - \frac{R^2 S^2}{A}$

Antibody No.	BVA		B 36		B 61		B 62		B 62		Serum 607		Serum 607	
Temperature, C.	37, 0		37, 0*		37, 37		0, 0		37, 37		0, 0		37, 37	
R.	13.6		12.4		11.4		(17)		12		(15)		(11)	
A.	4.08		1.86		1.71		(1.23)		1.20		(1.42)		(1.31)	
S III used	N pptd.		N pptd.		N pptd.		N pptd.		N pptd.		N pptd.		N pptd.	
	found	Calc.	found	Calc.	found	Calc.	found	Calc.	found	Calc.	found	Calc.	found	Calc.
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0.01							0.36	0.32						
0.02							0.57	0.59						
0.03			0.50	0.46	0.45	0.43	0.78	0.81	0.44	0.43	0.62	0.54	0.42	0.40
0.04					0.79	0.79					1.03	0.95	0.74	0.73
0.05	1.22	1.25	1.03	1.03	0.97	0.95	1.07	1.11						
0.06					1.08	1.09			0.96	1.01	1.25	1.23		
0.075			1.41	1.40							1.35	1.36	1.16	1.13
0.08					1.29	1.34								
0.09											1.40	1.42	1.23	1.23
0.10	2.24	2.27	1.66	1.65	1.54	1.52								
0.12					1.68	1.64								
0.20	3.62	3.62												
0.25	3.87	3.96												

R and A values in parentheses deduced from nearest actual determination.
*37,0 indicates that the tubes were incubated for 2 hours at 37° and placed in the refrigerator overnight.
From (20)

TABLE 4

Addition of Increasing Amounts of S III to 1.0 ml. of Rabbit-anti-Pneumococcus Serum diluted (1:1)

Amount S III added	Antibody N precipitated	Antibody N calculated from equation	Experimental ratio N precipitated to S III precipitated	Character of precipitate	Tests on supernatants
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>			
0.02	0.292	0.30	14.6	Op., Fl.	Excess A
0.04	0.554	0.54	13.9	Op., Fl.	Excess A
0.06	0.766	0.74	12.8	Op., Fl.	Excess A
0.08	0.866*	0.88	10.8	Op., Fl.	Excess A
0.10	0.926	0.97	9.3	Op., C.	Excess A
0.15	0.982*	0.98	6.5	Op., C.	No A or S
0.20	0.996			Excess S
0.50	0.940			Jelly	Excess S
1.00	0.602			Jelly	Excess S
1.50	0.362			Jelly	Excess S
2.00	0.336			Jelly	Excess S

Equation: Mg. antibody N pptd. = $16.1 S - 63.9 S^2$. S max. = 0.126 mg. A Max. = 1.015 mg. N

*One determination only.

Op = opaque; Fl = flocculent; C = compact
From (16)

TABLE 5

Addition of Increasing Amounts of Serum Albumin to Constant Volume of Antiserum

Sa N added	Sa N precipitated	Total N precipitated	Antibody N by difference	Ratio antibody N: Sa N in precipitate	Antibody N precipitated, calculated from equation [4]	Antibody N precipitated, calculated from equation [5]	Tests on supernatants
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>		<i>mg.</i>	<i>mg.</i>	
<i>Course 1. Rabbit 3.6%. 1.5 ml. serum used</i>							
0.025	Total	0.392	0.367	14.7	0.370	0.377	Excess A
0.038	Total	0.554	0.516	13.6	0.515	0.512	Excess A
0.050	Total	0.668	0.618	12.4	0.630	0.616	Excess A
0.080	Total	0.864	0.784	9.8	0.796	0.775	Excess A
0.094	Total	0.912	0.818	8.7	0.820	0.810	Excess A
0.100	Total	0.904	0.804	8.0		0.813	Trace A and Sa
0.113	Total	0.958	0.845	7.5		0.818	Trace A and Sa
0.125	Total	1.000	0.875	7.0			Trace A and Sa
0.150	0.144	1.010	0.866	6.0			Excess Sa
0.175	0.150	0.998	0.848	5.7			Excess Sa

Equation [4]: mg. antibody N pptd. = $17.0 Sa N - 88 (Sa N)^2$
Calculated from equation, Sa N max. = 0.0966 mg.

Antibody N max. = 0.820 mg.

Equation [5]: mg. antibody N pptd. = $22.4 Sa N - 45 (Sa N)^{3/2}$
Calculated from equation, Sa N max. = 0.110 mg.

Antibody N max. = 0.818 mg.

From (19)

TABLE 6

Addition of Increasing Amounts of Thyroglobulin to Homologous Antithyroglobulin Rabbit Serum

Tg N added	Total N precipitated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calculated from equation [4]	Antibody N calculated from equation [5]	Tests on supernatants
mg.	mg.	mg.		mg.	mg.	
Anti-hog Tg 12E ₁ serum 3.75						
Course 1. 2.0 ml. serum, hog Tg 13B ₂ B ₃ used						
Equation [4]: mg. antibody N pptd.=4.9 Tg N-15.2 (Tg N) ²						
Max. Tg N, A N=0.161, 0.395 mg. N respectively						
0.015(8)	0.098	0.082	5.2	0.074		Excess A
0.031(6)	0.170	0.138	4.4	0.140		Excess A
0.079	0.352	0.273	3.5	0.292		Excess A
0.158	0.562	(0.404)	(2.6)	(0.394)		Trace A, trace Tg?
0.316	0.804					Excess Tg
Course 2. 1.0 ml. serum, hog Tg 12E ₁ used						
0.158	0.556	0.398	2.5			Excess A
0.174	0.590	0.416	2.4			Excess A
0.248	0.720	(0.472)	(1.9)			No A, trace Tg?
Course 3. 1.0 ml. serum, hog Tg 12E ₁ used						
Equation [4]: mg. antibody N pptd.=5.5 Tg N-9.1 (Tg N) ²						
Max. Tg N, A N=0.302, 0.830 mg. N, respectively						
Equation [5]: mg. antibody N pptd.=8.2 Tg N-10.4 (Tg N) ^{2/3}						
Max. Tg N, A N=0.277, 0.756 mg. N, respectively						
0.032	0.230	0.198	6.2	0.167	0.202	Excess A
0.079	0.508	0.429	5.4	0.378	0.417	Excess A
0.158	0.784	0.626	4.0	0.642	0.641	Excess A
0.237	1.018	0.781	3.3	0.793	0.740	Excess A
0.316	1.168	0.852	2.7			Excess A
0.395	1.256	(0.861)	(2.2)			Trace A, trace Tg

From (6)

Table 7 lists data on the reaction between tobacco mosaic virus and rabbit and pig antiserum (27). This system is of particular interest in view of the size of the antigen. Combining ratios, expressed on a molar basis, ranged from 900 in the extreme antibody excess region to 450 in the equivalence zone (cf. table 2). The data of Beale and Lojkin (2) appear of doubtful validity because their sera were very weak.

TABLE 7

Precipitin Reaction of Tobacco Mosaic Virus

Antigen N added	Total N precipitated	Antibody N precipitated		Ratio of antibody to antigen in precipitate
		found	calculated from equation [4]	
mg.	mg.	mg.	mg.	
Pig antiserum. Equation: Ab N ppt. = 2.2 antigen N - 3.14 (antigen N) ²				
0.048	0.144	0.096	0.098	2.0
0.081	0.240	0.160	0.157	1.97
0.129	0.342	0.213	0.323	1.67
0.242	0.600	0.358	0.348	1.48
0.320	0.680	0.360	0.382	1.12
0.484	0.630	(antigen excess)
Rabbit antiserum. Equation: Ab N ppt. = 4.1 antigen N - 5.3 (antigen N) ²				
0.048	0.23	0.182	0.185	3.80
0.081	0.385	0.304	0.297	3.75
0.129	0.500	0.371	0.441	2.88
0.194	0.720	0.526	0.596	2.71
0.242	0.890	0.648	0.682	2.86
0.320	1.080	0.760	0.770	2.38
0.405	1.145	0.740	0.791	1.83
0.484	1.270	0.786	0.743	1.63

From (27)

OTHER QUANTITATIVE PRECIPITIN STUDIES

Malkiel and Boyd (27a) investigated the reaction between *Viviparus* hemocyanin and rabbit antisera. Since hemocyanin contains copper, specific precipitates could be analyzed for antigen by determining their copper content. The reaction between the d-glutamyl polypeptide, the capsular antigen of *B. anthracis*, *B. mesentericus* and *B. subtilis* (IV, 52), with horse and rabbit anti-anthrax sera has been studied by Ivanovics (27b). Perlman and Goebel (76) have recently studied precipitin reactions with the somatic antigens of dysentery bacilli.

CALCULATION OF THE TOTAL ANTIBODY N CONTENT OF AN ANTISERUM FROM THE EQUATION

Since $a = 2R$ and $b = \frac{R^2}{A}$ in equation [6] where R is the anti-

body N: antigen N ratio, and A is the amount of antibody N precipitated at the equivalence point (where all the antibody is precipitated), the total antibody content A of an unknown serum may be calculated. For example in the anti-Fa serum in table 1, using the value 15.8 for a and 83 for b, R equals 7.9 and A equals 0.75 which is in agreement with the experimental value. Alternatively, the total antibody content may be calculated by differentiating equation [3] with respect to x which yields the relation

$$\frac{d (\text{Ab N pptd.})}{dx} = a - 2bx \quad [8]$$

By setting the first derivative equal to zero and solving for x, the amount of antigen at the point of maximum precipitation equals:

$$x_{\max} = \frac{a}{2b} \quad [9]$$

In the case given (table 1) $x = 0.0952$. Substitution of this value in equations [4] or [6] yields

$$\text{Ab N max.} = 0.75$$

EXPERIMENTAL ESTIMATION OF THE TOTAL ANTIBODY N CONTENT OF AN ANTISERUM

It will be noted from table 1 and curve III, fig. 1, that the maximal amount of antibody N is precipitated from the serum at the point at which a small amount of antigen first appears in excess. The standard procedure is, therefore, to add antigen in slight excess in determining the antibody content of an antiserum. The amount of antigen to be added is determined by a preliminary experiment in which small amounts of antigen are added serially to 0.5 ml. of serum, and the precipitate is centrifuged off after each addition until no further precipitate forms. The total amount of antigen needed for the volume of serum to be used in the actual determination is then added to the measured volume of antiserum. Because of the Danysz phenomenon, i.e., that less antigen is required to precipitate a given amount of antibody when added in successive portions than when added at once, it is usually necessary to add slightly more antigen than that used in the preliminary serial additions. As a rule, inclusion in the total of the final serial addition which

failed to give a precipitate will serve to insure a slight excess of antigen. It is, however, necessary to test the supernatant from the determination to be sure that excess antigen is actually present and that the excess is not so large as to cause partial inhibition of precipitation. When larger amounts of serum are available, the preliminary test may be carried out more reliably by adding varying amounts of antigen to a number of 0.5 ml. samples of serum, centrifuging, and testing the supernatants for excess antigen. The amount of antigen found to give a *slight* excess is then used for the actual determination. Antibody N is calculated by subtracting the antigen N added from the total N of the washed precipitate. If only a slight excess of antigen is present in the supernatant, no significant error is introduced by subtracting the total antigen N added.

This procedure is, however, valid only in the case of single, pure antigens. It could not be employed, for example, to determine the total antibody in a rabbit antiserum to whole horse serum. Such a serum would contain anti-globulin as well as anti-albumin and when tested with whole horse serum would not yield a supernatant containing a slight excess of antigen and no antibody. Instead, there would be a region where both antigen and antibody are present in the same supernatant. It is therefore usually impossible to find a quantity of antigen which would precipitate the total amount of all antibodies present. A serum containing several kinds of antibody could be analyzed for total antibody only by carrying out separate analyses with one antigen at a time.

In the determination of total antibody by the quantitative agglutinin method, this difficulty does not arise. An antibacterial serum containing a variety of antibodies can be analyzed with a suspension of the bacteria provided the latter contain all the antigens in reactive form (I, 3).

ESTIMATION OF ANTIGENS

The quantitative precipitin technic affords an accurate method for the estimation of minute quantities of specific polysaccharides (28) and of small quantities of proteins (13). Determination of the amount of specific N precipitated in the region of antibody excess from a previously calibrated antiserum (one for which the curve of total N precipitated against antigen N added has been determined) permits the quantity of antigen in the sample to be read off from

the specific precipitate N curve. For example, if 1 ml. of a solution of Ea of unknown strength precipitates 0.53 mg. of N from 1 ml. of the anti-egg albumin serum (dilution = 1:1) described in fig. 1, the amount of Ea N in the unknown sample may be found to equal 0.04 mg. of N (curve IV, fig. 1). Sometimes, it may be necessary to employ a volume of antiserum other than the amount for which the curve has been plotted. The simple calculations necessary in such a case may be explained by example: If 3 ml. of an unknown solution of Ea precipitate 0.50 mg. of N from 2 ml. of antiserum (1:1), 1.5 ml. of antigen would precipitate 0.25 mg. N from 1 ml. of antiserum (1:1). This corresponds to 0.016 mg. of Ea N on curve IV, fig. 1. The unknown solution therefore contains 0.016 mg. Ea N per 1.5 ml. or 0.0107 mg. Ea N per ml. (Cf. II,7).

Since the amount of specific precipitate nitrogen measured is usually many times that due to the antigen, immunochemical determination of small amounts of antigen is more accurate than direct chemical analysis. The method is especially useful with mixtures provided these do not contain antigens which cross react with the antibody to the antigen to be determined. It has been applied to the estimation of albumin and globulin in body fluids by Goettsch and Kendall (29), and has been used as a guide for the isolation and identification of protein fractions from normal and pathological human sera by Kendall (30). A recent application to physiology has been made by Gellhorn (31), who determined blood volume in dogs, using pneumococcus type III capsular polysaccharide instead of the dye customarily employed. Details of these procedures are given in section II, chapter 7.

QUANTATIVE STUDIES OF VARIOUS FACTORS INFLUENCING THE PRECIPITIN REACTION

The development of the quantitative method made it possible to carry out precise studies of the effect of solubility, volume, time, temperature, salt concentration and pH on the precipitin reaction and to investigate its mechanism.

Effect of repeated washings: Complete removal of non-specific serum proteins is effected by two or three washings with 0.9 per cent saline as demonstrated by Heidelberger and Kendall (28). The amount of nitrogen in the specific precipitate is not altered appreciably by repeated washing with 0.9 per cent saline. As a general

rule, two washings suffice when the amount of antiserum employed does not exceed 3 or 4 ml; if it is larger, three washings are advisable.

The validity of results obtained by analysis of the washed specific precipitate could be demonstrated by analysis of horse antibody solutions containing a large proportion of specifically precipitable N (28). The values for antibody N calculated from the difference in N content between the supernatant and the original antibody solution were found to agree with those obtained by direct analysis of the washed precipitate.

The effect of volume: Experiments on the effect of volume upon the amount of antibody precipitated (table 8) show that changes in the concentration of antibody have almost no effect on the amount of specific precipitate. This does not contradict the requirements of the mass law from which Kendall's equation was derived, since the volume term cancels out in the theoretical derivations of the precipitin equations (20, 25).

TABLE 8
Effect of Volume on Total N Precipitated in 48 hours at 0° C

Species of anti-serum	Anti-serum to	No. of se-rum	Anti-gen used	Amt. anti-gen added	Total volume in ml.								Solu-bility	Data from	
					1	2	4	5	6	8	9	10			12
					Total N precipitated										
				mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg. N/ml.		
Horse	Pn III	B-62	SIII	0.03		0.87	0.91		0.87	0.84		0.84	0.87	(20)	
Rabbit	Pn III	3.49 ₁	SIII	0.10		0.550						0.494	0.007	(16)	
Rabbit	Pn III	3.50 ₁	SIII	0.15		0.796							0.736	0.006 (16)	
Rabbit	Pn III	3.51 ₁	SIII	0.06		0.496					0.474		0.003	(16)	
				<u>mg. N</u>											
Rabbit	Hen Ea	3.87 ₁	Ea	0.098		1.18					1.15		0.004	(13)	
Rabbit	Hen Ea	3.87 ₁	Ea	0.079		1.31				1.28			0.005	(13)	
Rabbit	Horse Sa	3.69 ₁	Sa	0.05		0.674				0.628			0.008	(19)	
Rabbit	Horse Sa	3.69 ₁	Sa	0.25		0.642				0.584			0.010	(19)	
Rabbit	Hog Tg	3.99 ₁	Tg	0.158	0.252		(0.254)	0.238		(0.246)			0.003 (0.001)	(6)	

Values in parentheses denote dilutions in normal rabbit serum; other dilutions in saline.

The solubility of specific precipitates of protein-anti-protein systems involving rabbit antibody is of the same order as that of polysaccharide-antibody precipitates. From experiments in the thyroglobulin system, it appears that the solubility in normal rabbit serum is less than that in saline. Comparison in the serum

albumin (Sa) system of the solubility of precipitates formed in the antibody excess zone and those in the antigen excess region revealed no differences.

In the egg albumin (13) and thyroglobulin (6) systems, solubility determinations were also made by variation of the volume of saline used for washing the specific precipitate. Solubilities were of the same magnitude as those obtained by dilution with saline before formation of the specific precipitate.

Although the solubility determinations cited are crude and the values reported indicate no more than order of magnitude, it is obvious that in precipitin analyses requiring the utmost precision, the volume in which the test is set up and the total volume of wash liquid should be kept constant. In the micro-modification of the quantitative precipitin analysis (32), solubility may be a significant factor (73) and will be discussed below.

Velocity of the precipitin reaction. Although it has been shown that the initial combination between antigen and antibody in the horse and rabbit-anti-Ea (15) and horse-antipneumococcus polysaccharide systems (33) does not take more than a few seconds, considerably more time is required for the complete particulation necessary for quantitative precipitation. With hyperimmune sera containing at least several tenths of a mg. of antibody N, two days at 0-5° C. are generally sufficient for complete precipitation. At 37° C. essentially quantitative precipitation in strong sera occurs in 1 to 2 hours, but with horse sera markedly less antibody precipitates at the higher temperature (20). Apart from temperature, the speed of precipitation depends a great deal on the strength of the serum. When the amount of antibody is less than 0.1 mg. per ml. of serum, it is usually necessary to allow more than 2 days for quantitative precipitation. For example, for analysis of convalescent sera from pneumonia patients (34, 35) a reaction time of about 1 week at 0-5° C. is necessary in addition to an initial period of 1 hr. at 37° C. Another factor which delays specific precipitation is the presence of active complement. Lastly, the speed of precipitation depends on the ratio in which antigen and antibody are mixed. Precipitation is generally most rapid in the region of the equivalence zone (the Ramon titration for diphtheria antitoxin is based on this effect; cf. experimental procedures) and slowest in the inhibition zone where in some instances a week or more may be required for complete preci-

pitation. It is therefore unsafe to assume that the standard conditions above are applicable in such instances, as is sometimes done.

Effect of temperature. In rabbit sera the amount of antibody precipitated at 0°C . is generally only slightly greater than that obtained at 37°C . (13, 16, 19). In horse sera, however, the difference is considerable and may amount to as much as 13 per cent at the point of maximum precipitation (20), so that experiments should be carried out at 0°C . for quantitative recovery of antibody. Precipitation in horse sera is not quantitative when 2 hours of incubation at 37°C . are followed by standing overnight in the refrigerator (36), a standard immunological procedure. In rabbit sera, however, these differences are not marked (12, 13, 16, 19). The effect of temperature may be very pronounced in cross reactions (37) (Cf. II, 9).

Salt concentration. A quantitative study has been made of the effect on the precipitin reaction of NaCl concentrations from 0.1 *M* to 1.79 *M* and of several other ions of higher valence (38). Some typical results with NaCl are given in table 9. It was found that increasing the salt concentration above 0.15 *M* brings about a reduction in specific precipitate nitrogen, so that, for example, only about 30 per cent of the total antibody was precipitated from a rabbit antibody solution to type III pneumococcus polysaccharide when the reaction took place in approximately 0.95 *M* NaCl. With horse antibody to type III pneumococcus polysaccharide the effect was somewhat less marked. The egg-albumin-rabbit antibody reaction was found to be far less sensitive to changes in salt concentration than the SIII-antibody reaction.

Comparison of NaCl, MgCl_2 and Na_2SO_4 showed no clear effect of a divalent cation or anion on the final result other than that of total ion concentration. The tetravalent ferrocyanide ion definitely counteracted the salt concentration effect.

The decreased precipitation of antibody at higher salt concentrations does not appear to be due to increased solubility but rather to a shift in the equilibrium between the reactants brought about by the presence of the salt, so that the same amount of antigen combines with a smaller quantity of antibody (38). This salt effect has been utilized to dissociate antibody from washed specific precipitates and agglutinates for the purpose of obtaining pure antibody solutions (Cf. IV, 43). The effect of salt concentration on the re-

TABLE 9

Effect of the Concentration of Sodium Chloride upon the Reaction between S III and Antibody, 37° and 0°

Final NaCl concentration	Horse antibody solution B 36					Rabbit antibody solution B 50*	
	0.1 M	0.15 M	0.51 M	0.93 M	1.79 M	0.15 M	0.93-0.98 M
S III used	Nitrogen precipitated						
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0.02	0.54	0.50	0.42	0.39	0.36		
0.05	1.13	1.03	0.90	0.84	0.75	0.43	0.24
0.075	1.41	1.41	1.29	1.15	1.03	0.60	
0.10	1.75	1.66	1.54	1.28	1.22	0.77	0.34
0.15	1.78	1.86	1.62	1.50	1.45	1.04	0.39
0.20†	1.82	1.85	1.70	1.58	1.51	1.18	0.41
Equations:							
mg. antibody							
N pptd.	27.5 S-104S‡	25 S-84S‡	22.2 S-72S‡	20.2 S-68S‡	18.1 S-57S‡	9.5 S-18S‡	5.0 S-15 S‡
A§.	1.82	1.86	1.71	1.50	1.44	1.25	0.42

* Prepared according to Felton.

† Excess S III

§ Calculated mg. antibody N pptd. at antibody excess end of equivalence zone.

From (38)

action between egg albumin and rabbit-antibody has been studied further by Oudin and Grabar (39). These authors found that washed egg albumin-anti-egg albumin specific precipitates could be dissociated partially by treatment with 15 per cent NaCl solution at room temperature. On dialysis against 0.9 per cent NaCl solution considerable precipitation occurred but the extract still gave a precipitin test on addition of a trace of egg albumin.

Effect of pH. Comparison of quantitative precipitin tests in the egg albumin-rabbit antibody system at pH 6.4 and 7.8 revealed no difference, even in the inhibition zone (13), in agreement with earlier studies within the same limits by Marrack and Smith (40).

Composition of the specific precipitate: In studies on the mechanism of the precipitin reaction, it became necessary to determine both antigen and antibody in the specific precipitate. Although much qualitative evidence could be marshalled to show that in the antibody excess zone all antigen is precipitated (Cf. ref. 13, page 709), the best proof emerged from studies of the dye-egg albumin (18) and thyroglobulin (6) systems, where the antigens contained chemical groupings (R-salt dye and iodine, respectively) which could be determined accurately, thereby making it possible to analyze specific precipitates both for total N and antigen. By subtraction of the latter in terms of N from total N, antibody N could be calculated. These quantitative studies showed in both the dye (18) and thyroglobulin (6) systems that all antigen is precipi-

pitated in the antibody excess and equivalence zones. Representative data for the thyroglobulin (Tg) system (6) were as follows:

Thyroglobulin added	Iodine in ppt.		Iodine recovered	Component in supernatant
	found	calculated		
<i>mg.</i>	<i>micrograms</i>	<i>micrograms</i>	<i>per cent</i>	
Hog Tg, containing 0.58 per cent iodine, with 1.0 ml. anti-hog Tg serum				
1.5	8.8	8.7	101	Antibody
2.0	11.1	11.6	96	Antibody
2.5	14.1	14.5	97	Trace Tg, excess antibody
3.0	16.4	17.4	94	Trace Tg, trace antibody
Human Tg, containing 0.62 per cent iodine, with 2.0 ml. anti-human Tg serum				
1.25	7.6	7.8	97	Trace Tg, excess antibody
1.50	9.3	9.4	99	Trace Tg, trace antibody
2.00	11.9	12.5	95	Excess Tg, trace antibody

There appears to be no reason to doubt that the same principle also holds true for other systems, like egg albumin-rabbit antibody, provided tests show the absence of antigen in supernatants. Where the solubility of the specific precipitate is considerable, part of the antigen would remain in the supernatant even in the antibody excess zone. The possibility of an appreciable solubility effect in microanalysis must be considered.

In the antigen excess and inhibition zones, analyses of specific precipitates for both antigen and antibody present no difficulty when the antigen contains a characteristic element or radical as in the case of dye-egg albumin or thyroglobulin. With a simple protein antigen like egg albumin, however, it is necessary to analyze the supernatant for excess antigen by setting it up in the antibody excess zone with a calibrated antiserum (cf. determination of antigen, II, 7).

When the reaction is partly inhibited by excess antigen, the supernatant contains free egg albumin as well as soluble compounds of egg albumin and antibody. The simple method of supernatant analysis is then no longer valid, since allowance must be made for the soluble compounds which will co-precipitate in the analysis of the supernatant for antigen. The method of calculation (13) given below, which takes this into account, gives results which appear valid far into the inhibition zone.

Let A = the maximum antibody nitrogen found in the serum, Ea = the amount of egg albumin N added, and N = the amount of nitrogen precipitated at the point considered. Then the amount of specific nitrogen (antigen as well as antibody) in the supernatant is given by $A + Ea - N$, and all of this nitrogen would be precipitated in the analysis of the supernatant for egg albumin with excess antibody (II,7) as was actually found in the dye-antidye system (18). The assumption is made that the entire precipitate obtained in this analysis is of uniform composition, i.e., that the soluble egg albumin-antibody complexes present can combine with antibody until their composition is the same as that of the specific precipitate formed by the free egg albumin present in its reaction with excess antibody. This is supported by the observation that the egg albumin-anti- Ea precipitate at the antigen excess end of the equivalence zone can combine with antibody added after precipitation is complete.

If N' = the nitrogen precipitated from the serum used in the analysis of the supernatant, and F = the fraction of the supernatant used in the analysis, $N' - F(A + Ea - N)$ = antibody N precipitated from the serum used in the analysis. If the curve of antibody nitrogen precipitated by Ea from this serum be constructed (as for example curve III, fig. 1), the amount of Ea corresponding to this quantity of antibody nitrogen may be read off. The percentage of egg albumin in this portion of the precipitate may then be calculated according to

$$\frac{Ea \cdot N \text{ found} \times 100}{Ea \cdot N \text{ found} + \text{antibody } N \text{ found}} = \text{per cent } Ea \cdot N.$$

Since it was assumed that the entire precipitate contains this proportion of Ea , N' times per cent $Ea \cdot N$ thus found $\div F = Ea \cdot N$ in the total supernatant, and $Ea \cdot N$ originally added *minus* this value = $Ea \cdot N$ in the original precipitate.

For example (tables I and 10), 0.124 mg. $Ea \cdot N$ precipitated 0.730 mg. N from 1.0 ml. diluted serum 387, $A = 0.750$ mg. N . Then $0.750 + 0.124 - 0.730 = 0.144$ mg. specific N in supernatant. When three quarters (F) of the supernatant (1.5 ml.) was set up with 1.0 ml. of the same diluted serum 0.412 mg. N (N') was precipitated. Then $0.412 - (0.75 \times 0.144) = 0.304$ mg. antibody N

TABLE 10
Calculation of Ea N in Precipitate in Region of Antigen Excess

(1) Ea N added	(2) Total N pptd.	(3) Specific N in su- pernatant (A+ Ea-N)	(4) Fraction analyzed	(5) Total N pptd. in anal- ysis of fraction	(6) Less specific N in fraction analyzed	(7) Corres- ponding Ea N	(8) Per cent Ea N in 2nd ppt.*	(9) Ea N in fraction anal- yzed	(10) Ea N in entire super- natant	(11) Ea N in ppt.
mg.	mg.	mg.		mg.	mg.	mg.		mg.	mg.	mg.
Serum 3.87 I, 1:1, maximum antibody N pptd., 0.750 mg.										
0.090	0.826	0.014	1.75	0.078	0.053	0.0035	6.2	0.0048	0.003	0.087
0.098	0.820	0.028	0.75	0.106	0.085	0.0057	6.3	0.0067	0.009	0.089
0.124	0.730	0.144	0.75	0.412	0.304	0.0219	6.7	0.0276	0.037	0.087
0.135	0.610	0.275	0.50	0.596	0.458	0.0257	5.3	0.0316	0.063	0.072
0.195	0.414	0.531	0.34	0.676	0.495	0.0395	7.4	0.0500	0.147	0.048
0.307	0.106	0.951	0.167	0.674	0.515	0.0418	7.51	0.0506	0.303	0.004

* 100 times-value in Column 7 divided by sum of values in Columns 6 and 7.

From (13)

pptd. from the serum used for the analysis. From Curve III in fig. 1 it is seen that this corresponds to 0.022 mg. Ea N, and

$$\frac{0.022 \times 100}{0.022 + 0.304} = 6.7 \text{ per cent. } 6.7 \text{ per cent of N', or } 0.412 = 0.028$$

and this divided by 0.75 = 0.037 mg. Ea N in the entire supernatant. 0.124 - 0.037 = 0.087 mg. Ea N in the original precipitate. The validity of this method was verified in the dye-Ea system by comparison with colorimetric antigen analyses on the original precipitate (18).

Calculations made as above in the region of excess antigen are given in table 10 in which the data in the first three columns are from table 1. The amounts of Ea N precipitated (last column of table 10), and the resulting ratios in the original precipitates are included in columns 2 and 5 of table 1.

COMPARATIVE UTILITY OF QUANTITATIVE AND QUALITATIVE PRECIPITIN TESTS

Apart from studies of mechanism or the accurate estimation of antigens, when it is indispensable, the quantitative method is generally capable of furnishing more information than qualitative tests. The degree of cross-reactivity between antigens of different species with a given antiserum can be established with precision, as in the study of various thyroglobulins (6). Alterations in sero-

logical properties due to chemical treatment of antigen can be followed in a precise way. For example, phosphorylation of horse serum albumin (41) was found to decrease the reactivity of this antigen with rabbit antiserum. The extent of the change in reactivity could be correlated with the phosphorus content of the derivative and with its viscosity in salt solution. A comparative study of egg albumin denatured in various ways revealed characteristic differences between egg albumin denatured by alkali on the one hand, and by acid or heat on the other (42). A marked difference between the response of egg albumin (43) and horse serum albumin (19) to coupling with the dye, R-salt, was found. The damaging effect of heat in the preparation of certain pneumococcus polysaccharides (44) escaped notice until such products were studied by the quantitative precipitin method using antisera from the rabbit. The value of R in equation [6], of a in equation [3] or of c in equation [5], are characteristic constants describing the combining properties of antibody with antigen. When either the antibody or the antigen is altered by chemical treatment, changes in the magnitude of R , a , and c may indicate the nature of the alterations (II, 10).

The determination of impurities in proteins or polysaccharides is discussed in chapter 8 of section II. The methods described there apply to contaminants which are serologically active. It is also possible to measure serologically inactive materials present in admixture with an antigen. For example, the relative purity of different lots of pneumococcus "C" substance may be estimated by analysis in the antibody excess zone using an antiserum calibrated with the most potent lot. In this way, it is possible to detect differences of only 5 or 10 per cent in potency in different lots which cannot be distinguished when tested qualitatively. Certain specific precipitates formed in the antibody excess zone may be analyzed for antigen and if it is found that not all the antigen added is in the precipitate, the amount of inactive material which remained in the supernatant may be calculated (cf. II, 8).

Univalent or incomplete antibody. Although it was assumed in the derivation of the quantitative theory (20) that the antibody in an immune serum is homogeneous the authors recognized that this was not strictly true. In the study of the egg albumin (13) and serum albumin (19) systems it could be shown, for example, that after removal of part of the antibody by precipitation with

antigen, the remainder possessed properties different from those of the unabsorbed serum, as evidenced by a change in the characteristic equation for the precipitin curve.

Another indication of the inhomogeneity of antibody emerged from the study of quantitative precipitin reactions by serial addition of small amounts of antigen to a portion of antiserum. It was found in the case of an anti-egg albumin serum, for example, that serial addition of antigen until no more precipitation ensued yielded only 78 per cent of the antibody precipitable by addition of the proper amount of antigen in one portion (13). The non-precipitable antibody (22 per cent of the total) was termed univalent or incomplete (45) since it was believed to be incapable of precipitation because each molecule of antibody possessed only a single group reactive

TABLE 11

Influence of Days between Last Injection and Bleeding, also of Repeated Bleedings, on Precipitin Content of Rabbit Sera

Rabbit No.	Total antigen injected	Total No. of injections	Weight precipitable antibody per ml. on day indicated after last injection						
			3rd	4th	5th	6th	7th	8th	10th
	mg.		mg.	mg.	mg.	mg.	mg.	mg.	mg.
	In solution								
5-5	18.7	18*				0.97		0.75	
6-8	13.2	16				1.22	1.04		
2nd course									
5-5	26.4	25*	0.79	0.61	0.39				
5-6	26.4	25*	0.36	≠†	≠†				
6-2	26.4	25*	0.83	0.85	0.47				
6-4	20.9	23	0.66	0.62					
6-8	20.9	23	2.06	1.88	2.04				
6-9	20.9	23	1.09	0.88	1.08				
3rd course									
5-5	53.9	32	≠		0.79		0.81		0.89
6-1	53.9	32	≠		0.94		0.86		0.80
6-2	53.9	32	≠		0.42		0.36		
	As alum precipitate								
8-9	14.5	18	2.03	1.91					
1-03	36.1	19			1.43	1.48			
1-12	28.4	32			0.95		0.75		
1-15	19.1	30			1.87 (test bleeding)				
					1.58 (main bleeding, same day)				
1-20	28.1	16			0.46		0.43		
	On collodion particles								
1-31	0.55	16			0.24		0.24		
1-33	0.55	16			0.54		0.23		

* All but two injections subcutaneous.

† Traces of precipitate in the 0.5 ml. samples used.
From (12)

with antigen, in contrast to precipitable antibody, which is held to be multivalent, i.e., possessing two or more reactive groups. Such univalent antibody can, however, co-precipitate with multivalent antibody and antigen, and it can thus be demonstrated and measured (13) (cf. I, 6). Such determinations are carried out as described below.

Study of the serum of a horse in the early stages of immunization with egg albumin revealed the presence of incomplete antibody at a time when there was no precipitable antibody present (15). Later bleedings from the same horse contained precipitating antibody of the antitoxin variety (see below).

Rate of antibody formation and breakdown in actively immunized animals. The quantitative precipitin method is ideal for following the level of circulating antibody at varying times after immunization. Variations in response among different individuals and differences between species as well as in the antibody-producing properties of various antigens may be compared with precision.

Data in table II (12) summarize antibody determinations on rabbit-antisera to R-salt azobiphenylazo-egg albumin taken 3 to 10 days after the final injection of azo protein. It is apparent that the maximal antibody content is usually reached by the third day except in rabbits which have received heavy doses in their final injections (3rd course of rabbits 5-5, 6-1, 6-2). When the immunizing doses are smaller, the level of antibody usually begins to decline about one week after the last injection. In a study extending from 10 to 20 days after the final injection of a rabbit immunized against type III pneumococcus it was found that the antibody content declined from 4.0 mg. to 1.3 mg. antibody N during this period (46) (fig. 2).

The experiment quoted involved the use of the isotope of nitrogen, N^{15} , to investigate the incorporation of dietary nitrogen into antibody protein. It was shown that antibody, like the other serum and body proteins, participates in metabolic reactions involving the uptake of dietary nitrogen. Although the feeding of isotopic glycine as a source of N^{15} was started on the 10th day after the final immunizing injection when the antibody level was actually declining, it was found that the N^{15} content of the antibody rose rapidly during administration of isotopic glycine, showing that

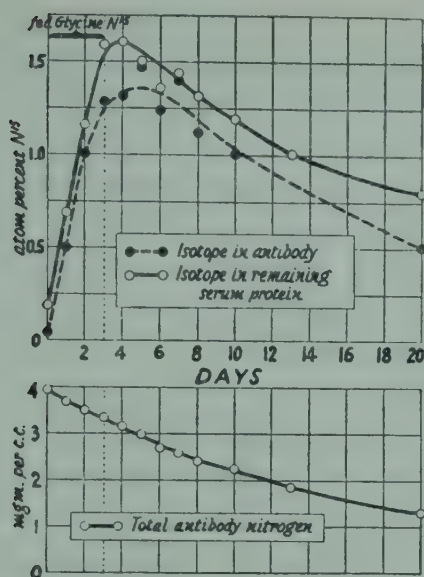


FIG. 2. Concentration of N^{15} in serum protein and antibody of immunized rabbit during and after the feeding of isotopic glycine (calculated for an isotopic content of 100 atom per cent in the compound administered). From (46).

antibody was being synthesized constantly although declining in concentration due to simultaneous breakdown at a rate exceeding that of synthesis. The rate of disappearance of N^{15} from antibody after termination of isotopic glycine feeding indicated a half-life of about 2 weeks for the antibody molecule.

Antibody which was transferred passively disappeared even more rapidly and did not take up N^{15} (47), indicating that in the absence of an active immune mechanism, antibody protein circulating in the body enters into metabolic reactions which lead to its disappearance, but not to uptake of nitrogen which may be considered to be associated with antibody synthesis.

The first quantitative study of the immune response of humans to immunization with pneumococcus polysaccharides was made recently by Heidelberger and collaborators (35) using the micro-precipitin method (32). One of the surprising results of this investigation was the long duration of the antibody response to 0.05 mg. of polysaccharide. Since antibody, like the other serum proteins, is subject to continuous synthesis and breakdown in an actively immune animal (46, 47), such a prolonged response is probably the result of continued functioning of the antibody-forming mechanism.

Relation between mouse protection and specifically precipitable antibody N: The demonstration by Heidelberger, Sia

and Kendall (48) that the protective power (52) of type I anti-pneumococcal horse sera was proportional to their content of specifically precipitable antipolysaccharide N, makes it possible in certain instances to substitute the more precise chemical method for the relatively crude and expensive animal assay or for the less precise relative methods (50, 51) of estimating potency. Further studies (49) showed that antipneumococcal horse and rabbit sera of various types could be standardized in a similar manner (I, 6).

TABLE 12

Addition of Increasing Amounts of A Substance to a Given Volume of Serum from Individuals of Blood Group O After Immunization with A and B Substances

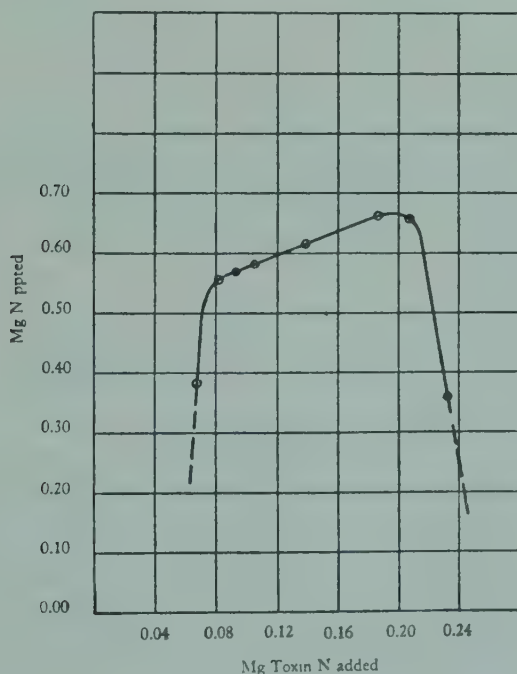
Amount A added	Antibody N ppted.	Tests on Supernatant	
		Hemagglutination for A cells	A substance in entire supernatant*
<i>micrograms</i>	<i>micrograms</i>		<i>micrograms</i>
1.0 ml. E. K. ₁ serum used—original titer 512, total volume 3 ml.			
25	18.1	32	0
50	37.2	8	0
75	43.6	2	0
100	47.6	1	0
150	55.8	0	1-2
200	58.5	0	15
0.5 ml. E. K. ₂ serum used—original titer 512, total volume 2.5 ml.			
10	9.2	16	0
30	21.5	4	0
50	25.3	2	0
75	27.4	0	0
100	26.8	0	2
150	28.2	0	6
1000	9.6		
Salt	0.0	64	
1.0 ml. F. P. ₁ serum used—original titer 128, total volume 2.5 ml.			
10	2.8	4	0
30	8.0	0	0
50	9.9	0	6
75	13.7	0	>12
100	10.8	0	>12
Salt	0.0	32	

* By inhibition of hemagglutination
From (53)

The quantitative precipitin method can thus be used to advantage when a purified antigen associated with protection can be obtained.

Precipitin tests with human isoagglutinins (53): The discovery that human blood group A substance prepared from hog gastric mucin gives a precipitin test with sera of normal and immunized humans of blood groups O or B made it possible for the first time to make quantitative measurements of isoagglutinating antibody. Since the amount of antibody is quite small even in sera of high titer, the highly sensitive micro-precipitin method developed by Heidelberger and MacPherson (32) was used. Representative data from (53) on the reaction of blood group A substance with homologous human antibody are given in table 12 and indicate complete precipitation of antibody at the point where blood group A substance first appears in the supernatant. For additional studies with blood group antigens cf. references 73 and 74.

Equine antibodies of the antitoxin type. The Ramon flocculation reaction between diphtheria toxin and horse antitoxin has long been known to differ from the precipitin reaction (fig. 1) in that the specific precipitate is soluble in an excess of either antibody or antigen. Washed toxin-antitoxin floccules were analyzed for



—Courtesy of Williams and Wilkins Company

FIG. 3. Flocculation of Diphtheria Toxin and Antitoxin. Data from (56).

nitrogen by Smith and Marrack (54) who demonstrated that non-specific protein was not carried down by the floccules, and by Healey and Pinfield (55) who showed that a flocculation unit (Lf) of toxin could combine with one or two units of antitoxin. The quantitative precipitin method was applied by Pappenheimer and Robinson to a study of the toxin-antitoxin reaction (56). Table 13 and fig. 3

TABLE 13

Addition of Increasing Amounts of Diphtheric Toxin to 300 Units of Antitoxin

A—antitoxin 621—300 units and 8.11 mg nitrogen per ml. T—toxin GH3-4—0.00055 mg nitrogen per Lf unit.

I	II	III		IV	V	VI
Lf units toxin	Toxin* nitrogen	Supernates¶		Nitrogen in precipitate	Antitoxin- Nitrogen in precipitate (IV—II)	Ratio A-nitrogen T-nitrogen (V:II)
		Toxin, total Lf	Antitoxin, total units			
	mg.			mg.		
50†	0.023		190§	0	0	
100†	0.046		95§	0	0	
150‡	0.069			0.386	(0.474)	(6.9)
175	0.081		Trace¶	0.554	0.473	5.8
200	0.092			0.564	0.472	5.1
225	0.103			0.579	0.476	4.6
300	0.138			0.612	0.474	3.4
400	0.184	Trace¶		0.661	0.477	2.6
425						(2.4)
450	0.207	Trace¶		0.652		
500‡	0.230	100§		0.359		
600†	0.276	240§		0	0	

* The nitrogen (column IV) precipitated by 200 Lf of toxin subtracted from that precipitated by 400 Lf and divided by 200 gives 0.00048 mg nitrogen per Lf of toxin. The figure used in column II, however, is 0.00046 mg nitrogen per Lf, the average obtained from six titrations including the above.

† No flocculation.

‡ Incomplete flocculation.

§ Determined by flocculation.

¶ By intracutaneous rabbit-test.

|| Average of duplicates.

Figures in parentheses were calculated for the ends of the neutral zone assuming 150 Lf and 425 Lf and complete flocculation.

From (56). Courtesy of Williams and Wilkins Co

show the type of data and curves obtained when increasing amounts of diphtheria toxin react with a constant volume of antitoxin. It will be noted (table 13) that no precipitation is obtained when 50 or 100 units of toxin are added to 300 units of antitoxin, and that a zone of flocculation occurs with larger amounts of toxin until a maximum of total N is precipitated when 400 units of toxin are added. Addition of still larger amounts of toxin results in a decrease

in total N precipitated, and finally in complete inhibition. Throughout the range of complete flocculation, neither toxin nor antitoxin could be detected in the supernatant by intracutaneous tests in rabbits, indicating complete precipitation of both toxin and antitoxin. The amount of toxin N corresponding to the number of toxin units added could be calculated from the increase in total N precipitated when 400 units of toxin were added as compared with 200 units of toxin. From table 13 it will be seen that this value for 200 Lf units of toxin amounted to 0.097 mg. N. One Lf unit of toxin therefore equals 0.00048 mg. N. As an average of six antitoxic sera the value 0.00046 mg. N per Lf unit of toxin was found, which is the value used in column 2 of table 13. Subtracting the toxin N from the total N precipitated gives the amount of antitoxin N in the precipitate and it will be seen in column 5, table 13, that this value is constant throughout the zone of complete flocculation and up to the point at which a very slight excess of toxin can be detected in the supernatant.

Purified preparations of diphtheria toxin have been obtained (IV, 50) in which values for N per Lf unit corresponded closely to those given above (56), indicating that these preparations were of relatively high purity.

For determination of the antitoxin content of a serum, it is only necessary to analyze the washed precipitates formed at two points within the flocculation zone, and to demonstrate the absence of toxin and antitoxin in the supernatant. The difference between the total N precipitated between the two points makes it possible to calculate the toxin N content of the specific precipitate and hence the antitoxin content may be calculated by difference. For example, if, in the equivalence zone, 2 and 4 ml. portions of an unknown toxin solution precipitate 0.564 and 0.661 mg. N respectively, from 1 ml. of the antitoxin serum given in table 13, $0.661 - 0.564 = 0.097$ mg. toxin N per 2 ml. Hence antitoxin N per ml. of serum equals $0.564 - 0.097 = 0.467$ mg. or $0.661 - 2 \times 0.097 = 0.467$ mg. (Note asterisk in table 13). As these calculations show, it is not even necessary to know the number of Lf units per ml. of toxin. Instead, results are obtained in terms of mg. toxin N per ml. toxin. Since the maximal antitoxic antibody N precipitable from 300 units of antitoxin was 0.47 mg., one unit of antitoxin equals 0.0016 mg. N. This method is applicable to crude as well as purified toxins.

Scarlatinal toxin gives similar types of curves with antitoxic sera (57). Pappenheimer (58) subsequently found that the toxin-antitoxin type of flocculation zone was shown by horse anti-egg albumin serum in its reaction with egg albumin. Pappenheimer's sera were also studied by Heidelberger and collaborators (15) who found that early bleedings which gave no flocculation contained univalent antibody. Hooker and Boyd (14) also found equine anti-hemocyanin sera to show a toxin-antitoxin type of reaction curve. Heidelberger, Treffers and Freund (59) found that injection of rabbit serum albumin subcutaneously into a horse resulted in antibody showing a toxin-antitoxin type of flocculation, while injection of rabbit serum globulin intravenously into another horse yielded antibody that gave a typical precipitin curve similar to those observed with rabbit antiserum. No precipitating or flocculating antibody was obtained in two horses, one of which was immunized with rabbit albumin intravenously and the other with rabbit globulin subcutaneously. These findings suggest that the route of immunization as well as the antigen used may be important in determining the type of antibody produced.

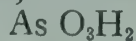
Precipitin reaction with polyhaptenic simple substances.

The foundation for the concept that the immunological specificity of antigens is intimately related to their chemical structure was laid by the work of Landsteiner (60) who coupled various chemical substituents to proteins and showed that antibodies produced against these artificial conjugated proteins exhibited reactions specific for the introduced groups. Since antibody was also formed against the protein part of the conjugated antigen, it was necessary to employ test antigens in which the same substituent was coupled to an unrelated protein. For example, if an azodye conjugated to horse serum was used for immunization the corresponding compound with chicken serum could serve as test antigen without reacting with the antibodies to horse serum which may have been formed. Landsteiner and van der Scheer (61) subsequently showed that precipitating test antigens could be produced by coupling resorcinol or tyrosine with two haptenic groups, although a protein carrier was still necessary for immunization. Thus it was demonstrated that simple substances could react to give precipitin tests. On the basis of the framework theory (21, 26) of the precipitin reaction, which considers precipitation as the result of the combination be-

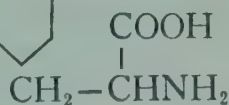
tween multivalent antigen and antibody, there appears to be no reason why simple compounds containing two or more haptenic groups should not precipitate with antibody in much the same way as conjugated proteins. These precipitating haptens offer the chemist an opportunity to investigate the mechanism of the reaction in more detail since the structure of at least one of the reactants, the antigen, is known. With this in view, an extensive series of quantitative precipitin studies was undertaken by Pauling and collaborators (62) using simple polyhaptenic substances as antigens. It was shown that compounds with two or more widely separated haptenic groups gave precipitin tests with the antisera while monohaptenic substances failed to react—evidence in support of the framework hypothesis (Cf. II, 9; for objections, however, cf (66)).

Inhibition of precipitation by simple substances. In precipitin reactions of certain dye-proteins with antisera it has been found that precipitation can be inhibited by addition of the dye-hapten or of related compounds. Numerous examples of such inhibition reactions have been described by Landsteiner (60) who used them to study relations between the chemical structure and serological specificity of haptens. For example, the reaction between

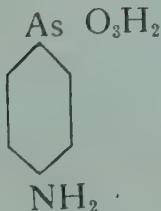
antibody to arsanilic acid-azo-horse serum

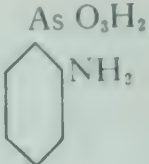



and the test antigen arsanilic acid-azo-chicken serum was found to be inhibited by arsanilic acid coupled to tyrosine,



by arsanilic acid itself,



by ortho-amino-benzene-arsonic acid 

and even by para-amino-benzene-sulfonic acid (67). 

Similarly, it has been observed that partial hydrolysis products of the specific polysaccharide (SIII) of the type III pneumococcus can inhibit precipitation of SIII with homologous rabbit antisera (62a). Avery, Goebel and Babers (68) prepared antisera to proteins coupled with the haptens p-amino-phenyl- α -glucoside and p-amino-phenyl- β -glucoside and found that precipitate formation by an antibody and the chicken antigen containing homologous hapten was inhibited only by the homologous hapten. Precipitate formation with the heterologous antigen, however, was inhibited by homologous and heterologous glucosides.

Inhibition reactions are attributed to the formation of soluble compounds between antibody and the non-precipitating simple substances. The effectiveness of an inhibitory substance is generally greater the more closely it is related in its chemical structure to the

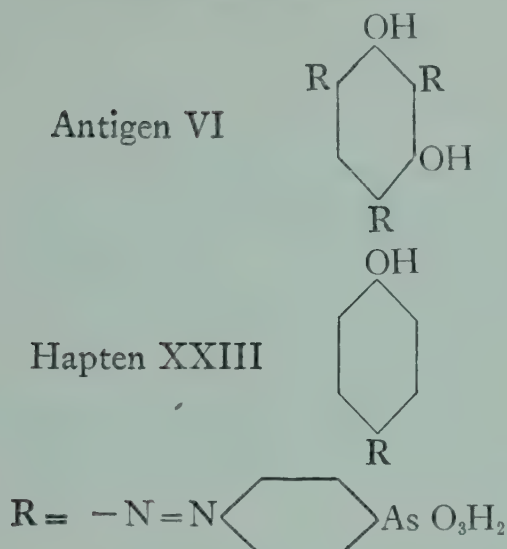
TABLE 14

Inhibition of Precipitation of Antiserum S and Antigen VI by Hapten XXIII

Antigen solution, hapten solution, and antiserum, 1 ml. each; 2 hours at room temperature, overnight in refrigerator. Blanks of antiserum and buffer: 0, 0, μ g. pH of all supernates 8.2.

Amount of hapten, μ g.	Amount of antigen, μ g.				
	6.3	12.5	25	50	100
Amount of antibody precipitated, μ g. ^a					
0	122	335	660	305	(144)
4.1	(131)	262	356	181	131
8.3	125	212	285	147	106
16.5	88	163	178	109	103
31	41	94	116	72	
63	9	41	31	35	(31)
125	6	6	19	12	12
250	0	0	3	0	3
500	0	0	6	0	3

^a Values are averages of duplicate analyses, with mean deviation of $\pm 8\%$ from the averages, except for the values in parentheses, which represent single analyses. From (62)



homologous precipitating antigen. As used by Landsteiner (60), the intensity of inhibition is estimated by performing a series of precipitin tests with constant amounts of precipitating antigen and antibody and varying quantities of hapten. The greatest dilution of hapten which causes inhibition may be taken as the titer. Precise determinations of the inhibitory effectiveness may be made by the quantitative precipitin method, as has been done by Pauling and collaborators (62). Illustrative data are given in table 14. It can be seen that the region of maximum precipitation with polyhaptenic compound VI does not shift significantly with increasing amounts of inhibiting monovalent hapten XXIII and that complete inhibition in the zone of maximum precipitation requires more of hapten XXIII than is needed in the region of antibody excess. Absolute values are rendered doubtful by failure to allow sufficient time for complete precipitation.

With antisera containing antibody of the antitoxin type inhibition also occurs with excess antibody. As in the case of inhibition of precipitation by excess antigen, inhibition of flocculation by excess antibody is due to formation of soluble compounds. This could be demonstrated in the horse-anti-Ea reaction (15) by the failure of rabbit-anti-Ea to precipitate when added to a non-flocculating mixture of horse-anti-Ea and Ea in the region of excess antibody and by ultracentrifugation in the toxin-antitoxin system (62b). It was also found in this instance that the soluble Ea-horse-antibody compound underwent slow dissociation with release of Ea,

which then could combine with rabbit antibody to yield a precipitate.

EXPERIMENTAL PROCEDURES

General considerations. The pH of antigen solutions should be near neutrality, i.e., within pH 6.5 and 8.5. The salt concentration should be near 0.15 *M*; otherwise non-specific precipitation or inhibition may occur. If the salt concentration departs considerably from isotonicity it may be adjusted by addition of water or solid salt or a concentrated solution of salt. In case the salt concentration is not known it is best to dialyze against 0.15 *M* NaCl solution.

Before the test, both serum and antigen solution should be centrifuged thoroughly to remove any particulate matter. Sera containing lipid may be centrifuged at 2000 or better at 10,000 r.p.m. for several hours in the cold until the fat rises to the surface when it can be removed by sucking it off. Coarse particles of lipid may be removed by filtration through absorbent cotton if they do not rise to the surface. The presence of lipid can usually be avoided by starving animals for a few hours before bleeding.

Each precipitin test should be accompanied by control tests, such as immune serum plus saline, normal serum plus antigen, or an unrelated immune serum plus antigen. No precipitation should occur in such control tubes. The possibility of bacterial contamination which may give rise to non-specific precipitates should be guarded against by observing sterile technic or by the use of preservatives like merthiolate (0.01 per cent final concentration) or phenol (0.2 to 0.5 per cent). If there is reason to question the specificity of a positive reaction, a stained smear of the precipitate should be examined for bacteria.

Negative tests require even more extensive verification. The potency of the antiserum or of the antigen should be checked. The possibility of inhibition by excess antigen or by excess antibody in the case of systems like toxin-antitoxin, should be considered. To do this the proportions of the reactants may be changed or a confirmatory test on the negative reaction mixture may be performed. For example, if a test for agar in an antigen suspected of containing agar is negative, a small quantity of agar should be added to the negative reaction mixture. If precipitation occurs, it may be concluded that the antigen is free from agar; if the confirmatory test remains negative, however, the original test was inhibited by excess

antigen or the antiserum used contained no antibody against agar (77).

When substituted proteins, like azo-dye proteins, are used as antigens, the presence of large amounts of free dye should be guarded against since they may cause inhibition. This difficulty may also arise with antigens which have been subjected to drastic treatment because breakdown products may be formed which could act as inhibiting haptens (62a) (Cf. II, 10).

The use of mixtures of antigens, like whole serum, in testing sera containing antibodies to several of the components usually gives rise to confusing results. Such difficulties may be avoided by fractionation of the mixture of antigens into its constituents and using only a single antigen at a time. In addition, sera containing several antibodies may be absorbed until only the desired antibody remains. For example, anti-pneumococcus sera often contain small amounts of antiprotein and anti-"C"-substance besides the antibody to the type-specific capsular polysaccharide. By absorption with an R-strain of pneumococci or with protein and "C" substance from a type other than that employed for immunization, the antisera may be rendered specific for the capsular antigen. Alternatively, the capsular polysaccharide may be freed from protein and "C" substance, but it is generally easier to absorb the immune sera (Cf. II, 11).

Qualitative test. A small volume of serum (0.10 or 0.5 ml.) is measured into a test tube. Antigen is added, and mixed with the serum, and the tube is incubated at room temperature or at 37° C. for $\frac{1}{2}$ to 1 hour, and placed in the refrigerator overnight. Appropriate blanks, i.e., serum plus saline, antigen plus saline, etc. are included. If antisera are potent, the tests may be read immediately after the initial incubation; if the sera are very weak, it may be necessary to wait several days. Read the tests by inspection for turbidity or a precipitate. If tests are negative or weak, centrifuge the tubes for 10 min. at 1500 r.p.m. and repeat the reading.

The quantity of serum to be used depends mainly on the antibody content. In general, about 0.1 to 0.5 mg. of antibody N will yield a satisfactory test, but if the potency of the antiserum is low, or if its amount is limited, as little as 0.01 mg. Ab N will yield a visible precipitate after centrifugation, provided enough time is allowed for the particulation of the antigen-antibody complex. When

antisera are very weak, it may be necessary to use as much as 4 or 5 ml. for a single test.

The choice of antigen quantity depends primarily on the amount of antibody in the test serum, i.e., it should be chosen so that the ratio of antibody to antigen is near equivalence. The main precaution to be observed is avoidance of inhibition by excess antigen. When the supply of serum is plentiful, much time can be saved by setting up a constant amount of serum with varying quantities of antigen, increasing in 2 or 3 fold steps. If, however, economy in the use of serum is necessary, it is advantageous to add only a small quantity of antigen initially, followed by larger doses added to the same mixture after a suitable period. For example, if an antigen solution of unknown strength is to be tested, one might start with 0.01 ml. If no reaction occurs in a few minutes (or a few hours or days, if the serum is weak), another 0.03 ml. followed by 0.1, 0.3, 1 ml. etc. may be added, allowing enough time for particulation after each addition. The process is, of course, one of trial and error, but much time and material can be saved by proper consideration of the potency of antisera, the strength of the antigen solutions and the combining ratios of antibody and antigen (cf. tables 3 to 7). After experience with a given immune system has been accumulated, there is usually little difficulty in evaluating these factors.

Supernatant tests. If a test is positive, the precipitate may be removed by centrifugation and portions of the supernatant may be tested for excess antibody or antigen, respectively. To test for excess antigen, a fresh portion of antiserum is added to the supernatant, while an excess of antibody is detected by addition of a small quantity (to avoid inhibition) of antigen.

Serum dilution titer. This is the classical method of assaying the potency of an antiserum. It involves setting up a series of progressive serum dilutions to find the limit of precipitation, i.e., the highest dilution at which a precipitate can still be observed. Titers obtained by this method, of course, depend on the physical conditions of the test, e.g., total reaction volume, time, temperature, method of reading (with or without centrifugation, etc.), but the most important factor is the amount of antigen used in the test. If a fixed quantity of antigen is employed, the proportions of antibody to antigen may vary from great antibody excess, through equivalence, to inhibition by excess antigen, as the antiserum is

progressively diluted. If the amount of antigen used is such that the endpoint of precipitation lies in the inhibition zone the titer observed depends on the amount of antigen used in the test as well as on the potency of the antiserum. For comparing the potency of a number of antisera in relative terms, the amount of antigen is not critical provided it is not present in large excess.

A procedure proposed by Martin (69) for selecting the amount of antigen to be used involves a preliminary titration with a constant volume of serum and progressively decreasing amounts of antigen. The least quantity of antigen which still yields a precipitate is then used with progressively decreasing amounts of serum. The variations in endpoint which occur with different amounts of antigen are illustrated with the data in table 15, in which a set of titrations with simultaneous variation of antigen and antiserum is given. It is seen that the endpoint titer of the serum is greatest with the least

TABLE 15
*Antigen Concentration Expressed in $\mu\text{g. per ml.}$
Direct Precipitation Tests*

Dilution of serum	256	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625
1:25	+	+	+	+	+	+	+	+	+	+	+	-	-
1:50	+	+	+	+	+	+	+	+	+	+	+	-	-
1:100	+	+	+	+	(+)	+	+	+	+	+	+	-	-
1:200	-	+	+	+	+	+	+	+	+	+	+	-	-
1:400	-	-	+	+	+	+	+	+	+	+	+	-	-
1:800	-	-	-	+	+	+	+	+	+	+	+	-	-
1:1600	-	-	-	-	-	+	+	+	+	+	+	-	-
1:3200	-	-	-	-	-	-	-	-	+	+	+	-	-
1:6400	-	-	-	-	-	-	-	-	-	-	+	-	-
1:12800	-	-	-	-	-	-	-	-	-	-	-	-	-
Supernatant Examinations													
1:25	A	A	-	a	a	a	a	a	a	a	a		
1:50	A	A	A	-	a	a	a	a	a	a	a		
1:100	A	A	A	A	(-)	a	a	a	a	a	a		
1:200	A	A	A	A	A	-	a	a	a	a	a		
1:400	A	A	A	A	A	A	-	a	a	a	a		
1:800	A	A	A	A	A	A	A	-	-	a	a		
1:1600	A	A	A	A	A	A	A	A	-	-	-		
1:3200	A	A	A	A	A	A	A	A	-	-	-		
1:6400	A	A	A	A	A	A	A	A	A	-	-		

+ Precipitate

- No precipitate

A Excess antigen in supernatant

a Excess antibodies in supernatant

(-) Optimal ratio (fastest reacting mixture)

From (69)

amount of antigen (the larger amounts of antigen cause inhibition), and that the titer (6400) obtained at this level of antigen corresponds to a mixture of antigen and antibody in equivalence zone proportions (see supernatant tests in table 15). It is also evident that the endpoint titer of antigen does not vary with the amount of serum used, indicating that the strength of an antiserum cannot be determined by a titration with progressive dilutions of antigen (cf. also 70, 71). For comparative titrations of different antigens, however, the antigen dilution method is useful (71), and in this case it makes little or no difference how much serum is used, provided the antibody is of the precipitin and not of the antitoxin type. For details of technic of dilution tests the procedure described for agglutination in I-3 may serve as a guide. The precision of dilution titers is at best subject to an error of a factor of 2.

Neutralization method. Since the equivalence zone ratios of antibody to antigen are characteristic of the immune system, a rough assay of the strength of an immune serum can be made by finding the amount of antigen necessary for equivalence (71). For example, it is seen in fig. 1 that the Ab N: Ea N ratios in the egg albumin system range between 9 and 10. Therefore, if 1 ml. of an unknown immune serum requires addition of 0.2 mg. egg albumin N for neutralization (i.e. neither antibody nor antigen present in the supernatant), it may be deduced that the antibody content is about 2 mg. of N per ml. Since combining proportions in the equivalence zone vary with individual sera and depend on the length of immunization (15), this method is at best only crude although its precision is greater than that of the serum dilution technic.

Procedure. A series of dilutions of antigen in two-fold steps is made by dilution from a stock solution of known concentration in terms of mg. antigen per ml. An arbitrarily chosen volume (e.g. 0.1 ml.) of each antigen dilution is mixed with 0.5 ml. of serum (undiluted or 1:2, 1:4, etc. depending on its potency), incubated for $1\frac{1}{2}$ hour at 37° C. and then put in the refrigerator overnight. After centrifugation on the next day the supernatants are decanted and tested for excess of antigen and antibody as described above. This series of tests should yield a crude indication of the equivalence zone. For a more precise determination a few tubes may then be set up in the vicinity of the equivalence zone using more closely spaced antigen dilutions. If the combining proportions of antibody

with antigen in weight units are known, results may be expressed in terms of mg. Ab N per ml., as illustrated above for the Ea-rabbit-anti-Ea system. The method is not applicable to antigen mixtures, like whole serum, since these do not yield a clear-cut equivalence zone.

Method of optimum flocculation or precipitation. The vicinity of the equivalence zone may also be located roughly by determining the ratio of antibody to antigen which flocculates most rapidly. The Ramon (63) flocculation test for diphtheria toxin makes use of this principle, as does the method of Dean and Webb (64) for determining the point of optimal proportions in precipitin systems (cf I, 1).

Estimation of the volume of specific precipitates. Estimations of the intensity of precipitin reactions have been made by measuring the volume of the specific precipitate (4, 72) but this method suffers from the variation in volume which accompanies different combining proportions of antibody and antigen.

Photoelectric turbidity measurements: A rapid and convenient turbidimetric method has been devised by Libby (75). Although it may be satisfactory for certain purposes such as the estimation of antigen or for use in systems where a single antigen is obtainable in relatively pure form, it is limited in that it is difficult to obtain precise comparisons of the potency of different sera and is affected by the numerous non-specific factors which influence rate of flocculation and size of particles.

Ring tests. The test is performed by layering the antigen solution carefully over a portion of antiserum in a narrow test tube. Appearance of turbidity at the interface constitutes a positive test. The use of this procedure is not generally recommended because it gives no information about combining proportions and because it is not readily reproducible. Its sole advantage over tests in which the reactants are mixed lies in the fact that inhibition does not occur so readily since the reactants diffuse into one another on standing.

Quantitative technic. To determine the total amount of precipitable antibody, set up a preliminary qualitative test, as described above, to find the amount of antigen which will be in slight excess. Measure a volume of thoroughly centrifuged serum which will yield about 0.5 to 1 mg. Ab N (as estimated from the preliminary test) in duplicate into test tubes (12 x 75 mm. for 2 ml. total volume; 12 or 13 x 100 mm. for 2 to 4 ml. total volume etc.)

using pipettes accurately calibrated to "blow out" delivery. A portion of serum to which saline is added instead of antigen is also measured as a blank. Add the amount of antigen which will provide a slight excess (this will give maximal precipitation in most systems) and close the tubes with rubber caps. The volume of antigen need not be measured with accurately calibrated pipettes. The contents of the tubes are carefully and thoroughly mixed by a rotary motion imparted by drawing the fingertips rapidly and repeatedly diagonally down the side of the tube.

If the test is to be performed in the cold, antisera, antigen solution and the tubes are chilled in ice-water and kept cold during all operations.

In tests requiring maximal precision, it is advisable to use not less than 1 ml. of antiserum. If this quantity would yield too much precipitate, the serum should be diluted accurately using calibrated pipettes and volumetric flasks. A convenient total volume for the test is 2 to 4 ml.; if necessary, saline is added as diluent, but it is well to keep the total volume the same in all tests, including the blanks, in order to avoid variations due to the slight solubility of specific precipitates.

With strong rabbit sera, almost all the antibody is generally precipitated within $\frac{1}{2}$ hour at room temperature or 37° C. Slightly greater recoveries may be obtained after 24 or 48 hours in the cold. If sera are weak, or in cross reactions and in the extreme antigen excess region of the inhibition zone, more time may be necessary.

After the tubes have stood long enough for complete precipitation, they are centrifuged from $\frac{1}{2}$ to 1 hour at 2000 r.p.m. in a refrigerated centrifuge. The tubes are removed from the centrifuge, immediately placed in ice-water and transported gently to the laboratory desk. The supernatant from each tube is carefully decanted by slowly inverting it with a continuous motion, holding it in the middle between the thumb and middle finger. (Placing the fingers near the bottom of the tube might warm the precipitate). During decanting, the supernatant should be observed closely to detect particles which may have become dislodged. If any are noted, the supernatant is recentrifuged to recover the lost precipitate. In the case of precipitates which do not pack firmly, it may be necessary to remove the supernatant by suction. If the precipitates are firmly packed, the tubes are kept inverted after decanting and allowed to drain for 1-2 min. on a clean towel. Complete

drainage is facilitated by using test tubes cleaned with dichromate-sulfuric acid. It is also advisable to maintain the original drainage channel by placing the tubes on the towel at a slight angle from the vertical leaning against a rack, with the original drainage channel downward. After drainage, the mouths are wiped with filter paper and the tubes are placed in ice-water. The packed precipitates are broken up by sharply tapping the tubes with the fingers. 2 to 4 drops of ice-cold saline are added and homogenized with the precipitate by repeated tapping. 0.5 ml. of cold saline is added and the contents are again mixed by tapping in order to insure thorough disintegration of the precipitate so that complete removal of non-specific protein is facilitated. The tubes are finally rinsed down with 1.5 ml. of cold saline for 75 x 12 mm. tubes (or with 2.5 ml. for 100 x 12 mm. tubes) while they are rotated with the tip of the washing pipette or by hand. The contents are again mixed. Complete wetting of the entire surface of the tube is necessary for efficient washing.

The tubes are allowed to stand in icewater for $\frac{1}{2}$ hour with occasional mixing. They are then centrifuged in the cold for $\frac{1}{2}$ to 1 hour at 2000 r.p.m. and the process of decanting, draining and washing is repeated once more. Two washings usually suffice unless the amount of serum exceeds 3 ml. or unless the tubes cannot be drained. After the final wash-liquid has been decanted, 1 or 2 ml. of water are added to each tube, the precipitates are tapped loose, and transferred quantitatively to a 100 ml. micro-Kjeldahl flask. (For estimation of colored antigens in specific precipitates, see below). If possible, the precipitate is kept intact rather than disintegrated so that its entire mass can at once be transferred into the Kjeldahl flask. A stirring rod is introduced into the tube and the transfer is completed by washing the entire inner surface with several drops of *N* NaOH and a few small portions of water.

When the precipitate does not pack very firmly, the initial supernatant is recentrifuged in a conical tube and the successive washings from the main tube are decanted into this conical tube. In this fashion all the washings are also recentrifuged in the conical tube. Finally an extra washing is given to the conical tube and its contents are transferred to the same Kjeldahl flask which received the main portion of precipitate.

Portions of the original supernatant are tested for excess antigen and antibody by addition of antiserum and antigen to $\frac{1}{3}$ and $\frac{2}{3}$ of the total supernatant, respectively. Tests are incubated and read

as described under qualitative tests. If there is a slight excess of antigen, it may be assumed that precipitation was maximal, and the amount of antibody N may be calculated without serious error by subtracting antigen N added from total N. Any nitrogen found in the serum blank should also be subtracted.

To determine the entire precipitin curve, increasing amounts of antigen are added to a constant volume of serum and the amount of precipitable N is measured by the procedure described above. A rough preliminary test to find the proportions of antibody to antigen in the equivalence zone will facilitate planning of the experiment. In the region of great antibody excess, where only a fraction of the total antibody is precipitated, analytical accuracy may be increased by using 2 or 3 times as much antiserum as near the zone of maximal precipitation, but when results are tabulated and plotted, all analytical values for the entire precipitin curve are recalculated to a standard volume of antiserum, usually 1 ml. Unlike the analysis for total antibody, where small variations in the amount of added antigen do not affect the total N precipitated, analyses for establishing the precipitin curve require precise delivery of antigen volume from calibrated pipettes.

Quantitative micro-estimation of antibody (32): The methods described above give their greatest degree of accuracy with quantities of antibody nitrogen ranging from 0.1 to 1 mg. There is frequently need, however, for a procedure which could be carried out with one-fifth to one-tenth these amounts, particularly in the case of human sera, in which the total antibody content in normal, diseased, or convalescent individuals is not likely to be large. The principal departures from the technic of the earlier method are precautions to ensure sterility during the relatively long interval between setting up the tubes and the washing and colorimetric estimation of the nitrogen in the precipitates. Depending upon the results of preliminary tests, three 1 to 4 ml. portions of serum are used.

Since heat inactivation may damage antibody in weak antisera (35), complement, which may add nitrogen to certain specific precipitates, is removed by addition of an antigen and the corresponding rabbit antibody unrelated to the immune system to be analyzed. These may be added either separately or in the form of finely divided specific precipitate suspended in saline. For analyses of antibodies to pneumococcal antigens of many types and of influenza bacilli, and meningococci, egg albumin (Ea) and rabbit anti-Ea are

used in this preliminary step. For 4.5 ml. of human serum, 0.04 mg. Ea N and 0.40 mg. of anti-Ea N have been recommended (65) for removal of complement. After centrifugation of the Ea-anti-Ea precipitate 48 hours later, the supernatant is divided into three equal portions, one of which serves as a blank. To the other two a slight excess of specific polysaccharide (usually 0.005 to 0.02 mg., as indicated by the preliminary tests) is added and the serum and solution are thoroughly mixed by rotating the tubes rapidly in a rack. Conical centrifuge tubes of about 8 ml. capacity are convenient to use. After $\frac{1}{2}$ to 1 hour at 37° C., the tubes are placed in the refrigerator for a week or ten days. During this period the contents are mixed twice daily by twirling in the rack. Centrifugation and washing in the cold are carried out as above. The blanks and precipitates are then taken up in water, treated with 0.2 to 0.3 ml. of 0.1 *N* NaOH until the precipitates are dissolved, and made up to 2.5 ml. or more, depending upon the amount of precipitate. Aliquots of 2.0 ml. are analyzed by the modified micro Folin-Ciocalteu method as described in (III, 22). Optical densities are read and converted into antibody N by means of a factor obtained with known amounts of antibody N, specific precipitate N, or normal gamma globulin N (IV, 38). The optical density is proportional to the amount of antibody N, but may differ for antibodies of different animal species and may not always be the same as that of normal globulin.

Quantitative determination of univalent antibody: A typical series of analyses is tabulated in table 16 to illustrate the determination of the amount of univalent antibody in an early bleeding from a horse immunized with egg albumin (15). In principle, the method consists in the formation of a known amount of specific precipitate in the presence of a given volume of serum containing univalent antibody and measuring the increase in total N precipitated. It was found that 1.01 and 1.02 mg. Ab N was precipitated from 1.0 ml. rabbit-anti-Ea serum by 0.11 and 0.12 mg. Ea N, respectively. With 0.14 mg. Ea N (column 7) 1.04 mg. total N was precipitated, but Ab N could not be calculated since there was excess antigen in the supernatant. In the presence of 1.0 ml. normal horse serum, 0.14 mg. Ea N deposited 1.08 mg. total N (column 8). The small difference between 1.08 and 1.04 was considered due to non-specific precipitation or experimental error. From the mixture of 1.0 ml. horse-anti-Ea serum and 1.0 ml. of rabbit serum (column

9), 0.14 mg. Ea N precipitated 1.37 mg. total N. Since there was no excess of Ea in this test, the entire amount of Ea N (0.14 mg.) plus the non-specific 0.04 mg. N found with normal horse serum, i.e., 0.18 mg. N, was subtracted from 1.37 mg. total N, yielding 1.19 mg. antibody N. Deducting 1.02 mg. rabbit Ab N from this value, yielded a value of 0.17 mg. univalent Ab N for the immune horse serum.

TABLE 16

Rabbit anti Ea pool <i>a</i> , <i>ml.</i>	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Horse 728 ₃ serum, <i>ml.</i>		1.0						1.0	1.0	1.0
Normal horse,*.....			1.0				1.0			
Ea N, <i>mg.</i>				0.11	0.12	0.14	0.14	0.14	0.16	0.18
Saline, <i>ml.</i>	2	1	1	2	2	2	1	1	1	1
N pptd., <i>mg.</i>	0.00	0.01	0.01	1.12	1.14	1.04	1.08	1.37	1.40	1.32
Less Ea N and blank.....				0.11	0.12			0.18†	0.20†	
Antibody N pptd., <i>mg.</i>				1.01	1.02			1.19	1.20	
Less rabbit antibody N.....								1.02	1.02	
Low grade horse antibody N.....								0.17	0.18	
Supernatants + Ea.....				—	—	—	—	—	—	—
Supernatants + anti-Ea pool <i>a</i>				—	±	++	++	—	+	±±

* From a horse injected with bovine tubercle bacilli. The serum contained no precipitins for bovine tubercle bacillus protein or carbohydrate fractions.

† The difference between the runs with and without normal horse serum, 0.04 mg. N, (columns 7 and 8) was added to the Ea N as a blank.

From (15)

Colorimetric determination of dye antigen (R-salt azo-biphenyl-azo egg albumin) in specific precipitates: After the final washing with saline the colored precipitate is suspended in water, dissolved by addition of 0.1 ml. of *N* NaOH, transferred quantitatively to a 5.0 ml. volumetric flask and made up to the mark with water. For the purpose of colorimetric comparison this appears to be the optimal amount of alkali for both precipitates and standards when the final volume is 5 ml. After the color estimation the solution is quantitatively rinsed from the colorimeter cup into a micro Kjeldahl flask. The 5.0 ml. colorimetric flask is also washed with water and the washings are transferred quantitatively to the Kjeldahl flask. As standard in the colorimetric comparison, a solution of the dye-protein of known concentration is used. Addition of colorless protein to the dye standard does not appear to be necessary.

Inhibition tests with haptens: In a preliminary test determine the amounts of antiserum and precipitating antigen which, when mixed will give a precipitate. Set up a series of tubes containing a constant volume of antiserum and add progressively increasing

amounts of hapten. Incubate for 30 minutes and add a constant quantity of antigen as determined in the preliminary test. Mix and incubate. The inhibition "titer" of the hapten is the smallest amount which inhibits precipitation completely.

For inhibition tests using the quantitative precipitin method, see table 14 and Pauling et al. (62).

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CHAPTER 3

AGGLUTINATION

Specific agglutination, or the clumping together of bacteria, erythrocytes, etc. in the presence of homologous antibody, has been considered by several investigators to be similar in nature to the aggregation or agglutination of bacterial suspensions by non-specific factors (1, 2). The immunochemist, however, has found it more useful to treat the process as a precipitin reaction taking place at the surface of large particles (3-6), such as bacteria, collodion, or carbon particles, erythrocytes, etc., and to consider the visible agglutination as an indication that the antigen particles are linked together by antibody molecules to form aggregates as a result of the combination of multivalent antibody with multivalent antigen on the particle surface (5, 6). Opposition to the concept of agglutination as a precipitin reaction at the particle surface centered about the oft-repeated observation that an immune serum could be diluted several thousand times and still cause visible agglutination, whereas precipitin reactions fade out when the anti-serum is diluted 1:10 to 1:50. From a consideration of the relative amounts of antigenic surface available for combination in the precipitin and agglutination reactions, Zinsser (3) was able to calculate that these observations do not necessarily conflict. He pointed out that on the intact bacterium the antigenic surface, if it were all available for combination with antibody, would be of the order of $1/10,000$ of the surface the same amount of antigen would possess in solution and many times more antibody would therefore be required to combine with the antigen in solution. Experimental evidence supporting this analysis was obtained by Jones (7) and by Pol (8) who showed that collodion particles coated with egg albumin were agglutinated by very high dilutions of anti-ovalbumin sera even though they gave precipitin reactions only at very low serum dilutions. Several investigators (9-11) have recently revived interest in this technic because its greater sensitivity permits the detection of very small amounts of antibody. The method has been used to demonstrate antibodies in the sera of patients with hypersensitivity to egg albumin (9) and to insulin (10), antibodies to tuberculin (11) and to several viruses in animal and human sera

(12) and in some instances to detect small amounts of antigen (13). Suspensions of *S. marcescens* coated with antigen have also been employed in a similar manner (13a).

The development of the quantitative chemical method for the estimation of agglutinins (14), makes it possible to measure the amount of antibody combined with agglutinated bacteria on a weight basis and to study and describe the course of bacterial agglutination as a function of antigen and antibody (5) as had been done for the precipitin reaction (6) (see I, 2). These investigations show that both agglutination and precipitation follow the same general type of equation, except for differences conditioned by the spatial limitations of a chemical reaction taking place at the bacterial surface. Agglutinin and precipitin to the type-specific polysaccharide of Type I pneumococcus (15) (see II, 11) were found to be identical.

For quantitative determination of agglutinins (14) accurately measured volumes of thoroughly washed bacterial (or other particulate) suspensions are added to measured volumes of antiserum. The increase in nitrogen content of the agglutinated bacteria after washing with saline (to remove non-specific serum protein) over that of an equal volume of suspension added to saline is measured. The detailed procedure is given below. To determine the total agglutinin content of an antiserum, the amount of suspension added must be sufficient to remove all the antibody. This is done by centrifuging off the agglutinated bacteria and adding another portion of suspension until no further visible agglutination occurs. Exhaustion may be confirmed by setting up an aliquot of the supernatant from the first determination with another portion of suspension and demonstrating that no additional antibody nitrogen is removed. With pneumococci, the amount of suspension to be used may also be calculated from the agglutinin titer (30) and similar data could presumably be obtained for other systems.

The validity of the method was demonstrated in several ways (14). The value obtained for the agglutinin content of an antiserum was independent of the presence of excess normal serum protein. Within experimental error the agglutinin content per ml. serum was independent of the amount of serum used for the analysis. By the use of purified antibody solutions, it was possible to measure both the increase in nitrogen content of the bacterial suspension and the decrease in nitrogen of the supernatant (after removal of the ag-

glutinated bacteria) and establish that washing the agglutinated bacteria did not involve losses of antibody.

If a quantity of bacterial suspension insufficient to remove all of the antibody was added, it was found that the amount of antibody N removed varied with the time of standing. Subsequent investigation showed that this variation was dependent largely on mechanical factors influencing the number of collisions of antibody molecules with the bacteria. If mechanical stirring or continuous shaking of the tubes was used, maximum combination with antibody occurred quite rapidly (5). Such treatment was found to be essential for precise studies on the mechanism of bacterial agglutination.

DESCRIPTION OF AGGLUTINATION IN QUANTITATIVE TERMS

The course of bacterial agglutination as a function of the amounts of bacterial suspension and antibody has been studied for the reaction of the type-specific capsular polysaccharide on Type I pneumococci with homologous antiserum (5). Increasing amounts of a suspension of Type I pneumococci (Pn I) of known N content were added to a series of tubes each containing a given volume of Type I antiserum or antibody solution. The tubes were shaken at the desired temperature until maximum combination of antibody with the pneumococci occurred, as determined by preliminary experiments. The tubes were then centrifuged at the same temperature, washed twice in the cold with saline and analyzed for nitrogen. Agglutinin N was obtained by subtracting bacterial N from total N.

Representative data obtained at 0° C. and at 37° C. using a suspension of pneumococci and a Felton antibody solution (see IV, 43) are shown in table I and figure 4. The polysaccharide content of the bacterial suspension used was determined as described in (II, 7). This information made it possible to calculate ratios of antibody N to polysaccharide over the course of the reaction (5).

It will be observed from table I and fig. 4 that the ratios reach an upper limit with decreasing amounts of suspension. This was also found to be true if increasing volumes of antiserum were added to a constant amount of type I pneumococcus suspension (Pn I).

In the region in which a change of ratio was observed, the general type equation derived for the precipitin reaction (I, 2):

$$\text{Antibody N removed} = 2RS - \frac{R^2}{A} S^2 \quad [1]$$

was found to hold. By plotting the ratios of agglutinin N to polysaccharide (S) against the S added, straight lines were obtained (lines A and B, fig. 4) from which the constants $2R$ and $\frac{R^2}{A}$ could be evaluated. The equations for the antibody solution at 37° and

TABLE 1

Addition of Increasing Amounts of *Pneumococcus* I S (M) Suspension to 1 Ml. of Serum or Antibody Solution at Various Temperatures and Salt Concentrations

Bacterial N	Equiva- lent S I content	Total N precipi- tated	Antibody N pre- cipitated	Ratio N: S in pre- cipitate	Antibody N calcu- lated from equation	Total N precipi- tated	Antibody N pre- cipitated	Ratio N: S in pre- cipitate	Antibody N calcu- lated from equation
mg.	mg.	mg.	mg.		mg.	mg.	mg.		mg.
Antibody Solution B 78, 0.15 M Salt, at 37°									
0.122	0.017	0.255	0.17	7.1*		0.218†	0.07	4.1*	
0.244	0.034	0.494	0.24	7.1	0.24	0.454	0.17	5.0*	
0.366	0.051	0.718	0.34	6.7	0.34	0.642	0.24	4.7*	
0.488	0.068	0.916	0.41	6.0	0.42	0.852	0.33	4.9	0.33
0.732	0.102	1.302	0.56	5.5	0.54	1.216	0.45	4.4	0.46
0.976	0.136	1.576†	0.59	4.3	0.59	1.574	0.56	4.1	0.56
1.220	0.170	1.840	0.61	3.6		1.880	0.62	3.6	0.63
1.464	0.204	2.076	0.60	2.9*		2.188	0.69	3.4	0.68
Serum, salt		0.014				0.036			
mg. antibody N pptd. = 8.0 S - 26.9 S ² S max. = 0.149 N max. = 0.594 calcd. 0.61 found									
Serum H 701, 0.15 M Salt, at 37°									
0.064	0.0165	0.254	0.18	10.9*		0.187	0.12	7.3*	
0.096	0.025	0.369	0.27	10.8	0.28	0.290	0.19	7.6*	
0.127	0.033	0.476	0.34	10.3	0.36	0.376	0.25	7.6*	
0.191	0.0495	0.686	0.49	9.9	0.49	0.534	0.34	6.9*	
0.255	0.066	0.868	0.61	9.2	0.59	0.722	0.47	7.1	0.47
0.382	0.099	1.060	0.67	6.8	0.72	1.030	0.65	6.6	0.63
0.509	0.132	1.202	0.69	5.2		1.244	0.74	5.6	0.74
0.637	0.165	1.340	0.70	4.2*		1.414	0.78	4.7	0.79
Serum, salt		0.008				0.000			
mg. antibody N pptd. = 12.5 S - 53.2 S ² S max. = 0.1175 N max. = 0.734 calcd. 0.70 found									
Antibody Solution B 78, 0.15 M Salt, at 0°									
0.122	0.017	0.255	0.17	7.1*		0.218†	0.07	4.1*	
0.244	0.034	0.494	0.24	7.1	0.24	0.454	0.17	5.0*	
0.366	0.051	0.718	0.34	6.7	0.34	0.642	0.24	4.7*	
0.488	0.068	0.916	0.41	6.0	0.42	0.852	0.33	4.9	0.33
0.732	0.102	1.302	0.56	5.5	0.54	1.216	0.45	4.4	0.46
0.976	0.136	1.576†	0.59	4.3	0.59	1.574	0.56	4.1	0.56
1.220	0.170	1.840	0.61	3.6		1.880	0.62	3.6	0.63
1.464	0.204	2.076	0.60	2.9*		2.188	0.69	3.4	0.68
Serum, salt		0.014				0.036			
mg. antibody N pptd. = 5.7 S - 11.6 S ² S max. = 0.246 N max. = 0.70 calcd. 0.69 found									
H 701, 0.15 M Salt, at 0°									
0.064	0.0165	0.254	0.18	10.9*		0.187	0.12	7.3*	
0.096	0.025	0.369	0.27	10.8	0.28	0.290	0.19	7.6*	
0.127	0.033	0.476	0.34	10.3	0.36	0.376	0.25	7.6*	
0.191	0.0495	0.686	0.49	9.9	0.49	0.534	0.34	6.9*	
0.255	0.066	0.868	0.61	9.2	0.59	0.722	0.47	7.1	0.47
0.382	0.099	1.060	0.67	6.8	0.72	1.030	0.65	6.6	0.63
0.509	0.132	1.202	0.69	5.2		1.244	0.74	5.6	0.74
0.637	0.165	1.340	0.70	4.2*		1.414	0.78	4.7	0.79
Serum, salt		0.008				0.000			
mg. antibody N pptd. = 8.8 S - 24.5 S ² S max. = 0.180 N max. = 0.79 calcd. 0.78 found									
For serum H 702 the equations were:									
0.15 M salt: At 37° , mg. antibody N pptd. = 6.9 S - 16.5 S ² S max. = 0.209 N max. = 0.72 calcd. 0.74 found									
2 M salt: At 37° , mg. antibody N pptd. = 7.7 S - 44.8 S ² S max. = 0.086 N max. = 0.33 calcd. 0.40 found									
At 0° , mg. antibody N pptd. = 5.8 S - 11 S ² S max. = 0.263 N max. = 0.76 calcd. 0.83 found									
At 0° , mg. antibody N pptd. = 6.3 S - 20.7 S ² S max. = 0.152 N max. = 0.48 calcd. 0.53 found									

* Points not considered in calculating equation.

† One determination discarded.

‡ Only two points not at maximum ratio available for calculation.

From (5)

at 0° are given in Table 1. Values for antibody N calculated from these equations were in close agreement with those found experimentally (Table 1). Similar equations, but with different constants, were obtained when the reaction was carried out in $2M$ NaCl (5).

In its quantitative aspects agglutination of Pn I was found to differ in three respects from the precipitin reaction (5). The antibody N:S ratios (table 1) reached a value in the region of excess antibody, beyond which no more antibody could be taken up by the bacteria. The value of $2R$ from the equations was found to be about 1.2 times this experimentally determined ratio (5).

In agglutination the combining ratios of the components are also found to be lower than those in the precipitin reaction. Since it is not known whether all of the type specific polysaccharide (S I) found in the Pn I is available for reaction, as is assumed in calculating the ratios given, these values have but limited significance. If, however, under a given set of conditions, the same fraction of S I reacts throughout the whole range, the entire set of ratios would differ only by a factor inversely proportional to the fraction reacting.

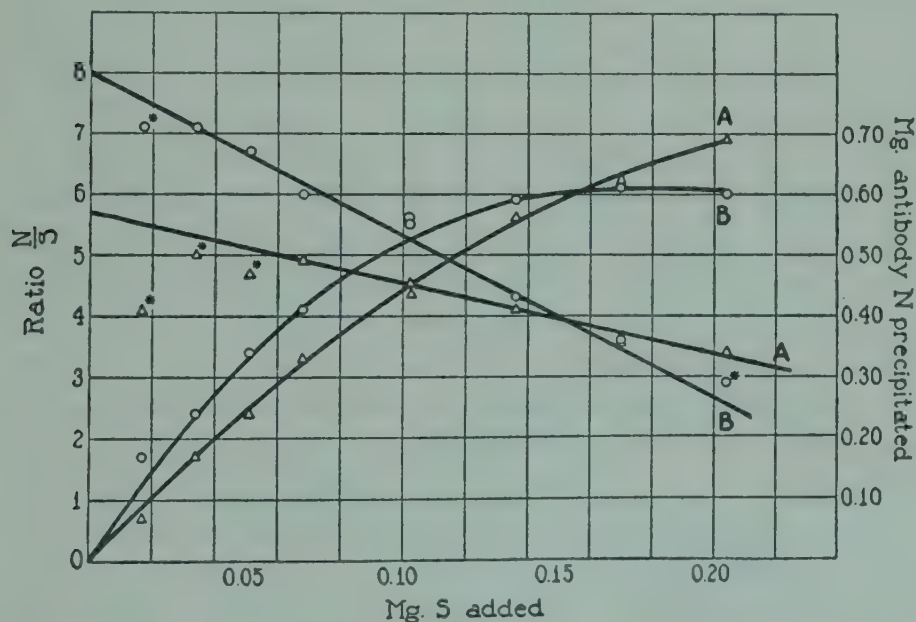


FIG. 4. Agglutinin N removed by increasing amounts of pneumococcus suspension from antibody solution B 78 in $0.15 M$ NaCl solution. Curve A and line A at 0° . Curve B and line B at 37° . From (5).

The third difference observed in agglutination (table 1 and fig. 4) is that throughout the region of antibody excess more antibody is

taken out at 37° than at 0° . If, however, the reaction is carried out first at 37° and then at 0° , the same quantity of Pn I suspension removes an even larger amount of antibody N. These results were taken to indicate (5) that a larger proportion of the S I in the organisms was capable of reacting at 37° than at 0° , but that after it has once reacted, continuation of the process at 0° takes place much as in the precipitin reaction, with more antibody removed at the lower temperature.

With excess Pn I, however, more antibody N is removed at 0° than at 37° .

Applicability of quantitative agglutinin methods: The quantitative agglutinin method makes possible an exact analytical determination of the total agglutinin content of antisera on a weight basis. This has generally been a measure of the total antibody content of the antisera, but it is conceivable that, in some instances, certain cell antigens may not be present at the surface of the bacterial cell and thus the organisms would not necessarily combine with antibody to these constituents. No well-defined instance of this kind has yet been reported among the organisms studied by quantitative procedures. Antisera to S and R (Dawson M and S (15b)) pneumococci (14-15a), hemolytic streptococci (16), hemophilus influenzae B (17), meningococci (18), gonococci (19), dysentery bacilli (19a), *B. proteus* (19b), *E. typhosa* (19c) and brucellae (20) have thus far been assayed by this technic. With some of these organisms, it is necessary to make the pH of the serum slightly acid to avoid dissolving nitrogen from the bacterial suspension. There is little doubt that the quantitative agglutinin method could be used with numerous other microorganisms.

In studies on type-specific antipneumococcal sera quantitative agglutinin estimations with suspensions of R pneumococci (cf. II, 9) provided data on the amounts of group specific antibody in these sera (15a). The method also yielded evidence that various ways of killing the bacteria, such as heat, formalin, or acid, can effect changes in antigenicity. Table 2 shows the amount of agglutinin N removed by Type I and II suspensions of heat, acid, or formalin killed organisms from one ml. samples of a rabbit antiserum prepared by injection of formalin killed IIR pneumococci (15a). Formalin killed organisms removed more antibody N than did heat or acid killed pneumococci and the data also show that a type-specific

TABLE 2

Antibody N removed from Antipneumococcus R Rabbit Sera by R Pneumococcus Suspensions

Rabbit Serum No.	Pn antigen injected	Antibody nitrogen removed per ml. serum by pneumococcus R (S)					
		Type I FK	Type I HK	Type I AK	Type II FK	Type II HK	Type II AK
1.76	II R FK	0.70	0.30	0.26	1.23*	0.64	0.37

FK = formalin-killed

HK = heat-killed

AK = acid-killed

* Serum not entirely exhausted after 3 to 4 absorptions.

From (15a)

antigen is present in the Type II R organisms. This antigen was not the Type II specific carbohydrate which was absent in the Type II R strain (15a). It is obvious that such definitive results could hardly have been obtained by the less precise qualitative agglutination procedures.

With hemolytic streptococcus suspensions, the method has yielded information about the type-specific and group-specific antigens of this organism. Thus table 3 shows results of quantitative agglutinin estimations on antisera to Type 1 and 3 hemolytic streptococci with Type 1 and 3 strains. The type-specific antibody content of the sera, computed by difference, is shown in the last column of the table (16).

Differences in cross reactivity among the Neisseria and individual differences among strains of *N. gonorrhoeae* are demonstrated in table 4 (19).

The method also may be used for the detection of small amounts of a given antigen in a microorganism. For instance, before quantitative studies on the identity of agglutinin and precipitin (15) could be carried out, it was necessary to absorb the group specific antibodies from the antisera with suspensions of R pneumococci. Since the sera were to be absorbed with a strain of R organisms derived from homologous type S organisms, it was necessary to know whether or not the R strain contained residual traces of type-specific carbohydrate. If a given R strain did contain even small amounts of type specific substance, it should be possible to remove all of the type-specific antibody from homologous type-specific

TABLE 3

*Agglutinin N Estimations on Rabbit Antisera to Streptococcus Hemolyticus,
1.0 ml. of 3:10 Serum Dilution Used*

Serum	Strain used for immunization	Agglutinin N per ml. of undiluted serum taken out by			Type-specific agglutinin N†
		SF 130, gl. Type I	Type 3 M	SF 130, R*	
		mg.	mg.	mg.	mg.
750 ₁	SF 130, gl.	0.29	0.16		0.13
750 ₂	SF 130, gl.	0.42	0.26	0.42	0.16
750 ₃	SF 130, gl.	0.35	0.22		0.13
751 ₁	SF 130, gl.	0.23	0.09		0.14
752 ₂	SF 130, gl.	0.41	0.25	0.50	0.16
752 ₃	SF 130, gl.	0.47	0.37		0.10
753 ₁	SF 130, gl.	0.29	0.15		0.14
756 ₁	SF 130, gl.	0.23	0.12		0.11
756 ₂	SF 130, gl.	0.40	0.32	0.43	0.08
756 ₃	SF 130, gl.	0.51	0.36		0.15
758 ₁	Type 3	0.08	0.35		0.27
758 ₂	Type 3	0.17	0.49	0.26	0.23‡
759 ₁	Type 3	0.28	0.36		0.08
759 ₂	Type 3	0.09	0.29	0.07	0.20
759 ₃	Type 3	0.27	0.51		0.24

Subnumerals indicate course of injection. Sera from the first two courses were given two absorptions only; agglutinin N removal was probably incomplete in 750₂, 752₂, 756₁, 756₂, and 758₂. Sera from the third course were given three absorptions, of which the first two sufficed for serum 750₃. Saponin was added to supernatants to prevent film formation.

* Suspension prepared from R strain; broth culture showed typical properties of R, but smears indicated possible presence of 5 to 10 per cent of S organisms.

† Homologous agglutinin N minus heterologous agglutinin N. Both include the anti-C.

‡ Higher value for heterologous agglutinin N used.

From (16)

serum by a series of absorptions. Table 5 shows the results obtained with Type I and III R suspensions (15). After the first absorption, the I R strain failed to remove any measurable amount of nitrogen from the serum although it still contained about 1.20 mg. Type I anticarbohydrate N per ml. Since the limits of error in the quantitative agglutinin method are about ± 0.02 mg. of nitrogen and since 0.01 mg. of Type I specific polysaccharide removes about 0.20 mg. of antibody nitrogen, 0.02 mg. of antibody nitrogen would correspond to about 0.001 mg. of polysaccharide. Inasmuch as the 0.44 mg. of bacterial N used for absorption failed to remove any antibody N from the serum it appeared that this amount of bacterial N contained less than 0.001 mg. of type-specific polysaccharide.

TABLE 4

Per Cent of Heterologous Agglutinin Nitrogen in Antineisserian Rabbit Serum as Measured by Quantitative Agglutination

Antigen	Neisseria gonorrhoeae				Neisseria intracellularis	Neisseria sicca	Neisseria catarrhalis
	#7-434 total agglutinin N 0.96 mg./ml.	#7-433 total agglutinin N 0.44 mg./ml.	#5-383 total agglutinin N 0.71 mg./ml.	#8-128 total agglutinin N 0.32 mg./ml.	#1-466 total agglutinin N 0.98 mg./ml.	#41-281 total agglutinin N 0.52 mg./ml.	#26-112 total agglutinin N 1.21 mg./ml.
Neisseria gonorrhoeae							
#7	100	100	76	31	18		
#5	43		100	38	35		3
#8	25	28	35	100	29	17	9
#2	32			75			
#9	56	59	66*		37	10	10
#10	51	50	82*				
#1	27	27	71	84		8	
#6					32	0	
Neisseria intracellularis							
Group I P.D.					100		
Group I #69	52	48	51		100	8	
Group II #38	28	25	25	42			
Group II Pe		37			47	6	7
Neisseria sicca							
#41	17	17	26	19	8	100	3
#2275			8*			0	3
Neisseria catarrhalis							
#26	0		0*			8	100
#20		14			4	5	56

* Values obtained from heterologous antigenococcal serum 382 with a total homologous agglutinin nitrogen content 0.67 mg./ml.

From (19)

The Type III R strain, on the other hand, continued to remove small amounts of antibody N in repeated absorptions and a limit was not reached. Using a weaker serum R₃₄₈, the total agglutinin contents as determined with a III R and III S strain were found to be the same (table 5). These data indicated that no significant amount of Type I specific polysaccharide was present in the I R strain, but that the III R strain did contain small amounts of Type III carbohydrate.

Detailed illustrations of the combined use of both the quantitative

TABLE 5
*Absorption of Agglutinins from Horse and Rabbit Antisera with Pneumococcus
I R, III R, and III S Suspensions*

Absorption No.	1.0 ml. H 610 (Type I S) horse serum	4.5 ml. H 53300 (Type III S) horse serum		1.0 ml. R 348 ₄ (Type III S) rabbit serum	
	Agglutinin N removed by IR	Agglutinin N removed by III R*		Agglutinin N removed by III R	Agglutinin N removed by III S
	mg.	mg.		mg.	mg.
1	0.16	0.14		0.098	0.176
2	0.00	0.08	Aliquot of 1st supernatant	0.070	0.022
3		0.06	Aliquot of 2nd supernatant	0.014	
4		0.06			
5		0.05	Total aggluti- nin N	0.182	0.198
6		0.05			
7		Absorption dis- continued			

In absorptions 2 to 7 on serum H 53300 the entire supernatant from the preceding absorption was used.
* Centrifuged sediment from 2.0 ml. suspension. Agglutinin N determinations with III R and III S suspensions gave 0.56 and 0.54 mg. N per ml., respectively.
From (15)

precipitin and agglutinin methods in studying bacterial antigens are given in II, 11.

Several modifications of the quantitative agglutinin method have been introduced using particulate suspensions other than bacteria. For instance, the antibody content of antisera containing hemolysins may be determined by the use of suspensions of washed sheep stromata (21). Eagle has used suspensions of the flocculating antigens usually employed in serological tests for syphilis, to measure the amounts of Wassermann antibody in human syphilitic sera (22). Volumes of serum of about 40 ml. were required and values of from 0.08 to 0.58 mg. antibody N were found in these samples. Davis, Moore, Kabat and Harris (23) measured the decrease in nitrogen in the supernatant by the Folin-Ciocalteu tyrosine method after removal of antibody from purified solutions of Wassermann antibody by Kahn floccules and obtained an estimate of the antibody content of these solutions.

Treffers and Heidelberger (24) have prepared antisera to specific precipitates and have used suspensions of washed antigen-antibody precipitates to study the specificity of antibody as an antigen with the antibodies in these sera (see I, 6).

Limitations of the quantitative agglutinin method: The chief limitation of the quantitative agglutinin method is the dependence of the method upon measurement of differences in nitrogen. Each value for agglutinin nitrogen involves two sets of nitrogen analyses. Since the nitrogen content of the organisms used is usually comparatively high, larger errors will result when the amount of antibody nitrogen removed by a given amount of bacterial nitrogen is but a small fraction of the added N of the organisms. If the limits of the error in the method are ± 0.02 mg. N and 0.20 mg. of bacterial N removed 0.8 mg. antibody N, the antibody N content will be 0.80 ± 0.02 or ± 2.5 per cent. If, however, 0.20 mg. suspension N only removed 0.02 mg. antibody N, the error will be 0.02 ± 0.02 or ± 100 per cent. Therefore, with suspensions of microorganisms which can take up only a very small fraction of their own weight as antibody, the method can not be used with precision, since numerous absorptions are required to determine the total agglutinin content and the summation of errors would make the final result very uncertain. This occurred to some extent with suspensions of hemolytic streptococci (14, 16) so that the values for agglutinin N were less accurate, although the method could still be used. With intact erythrocytes, however, the amount of antibody N removed is so small a fraction of the erythrocyte N that the method is impractical. By preparing stromata suspensions and eliminating the immunologically inert hemoglobin N, the method may be used with moderate precision (21). Before a new system can be investigated by the quantitative agglutinin method, it is necessary to establish that a sufficiently high proportion of antibody N to bacterial or other particulate N will be removed.

From the above discussion, it is also apparent that the quantitative agglutinin method is not satisfactory for measuring the agglutinin N content of very weak antisera, i.e., sera containing less than 0.01-0.02 mg. antibody N per 5 ml. serum, unless very large volumes of serum are available for analysis (cf. 22).

It should also be recognized that the quantitative agglutinin method generally measures the total antibody content of an anti-

serum. Since antisera prepared to intact bacteria, culture filtrates, etc., usually contain antibodies to several antigens in varying proportions, it is by no means certain that the total agglutinin N will correlate well with activities associated with only one of these antibodies, e.g., mouse protective power in antibacterial sera, Forssman antibody in antisera to sheep erythrocytes etc., unless the proportions of the inactive antibodies are but a small fraction of the total antibody N. This is generally the case in type-specific antipneumococcal sera.

TABLE 6

Protective Potency of Horse (H), Rabbit (R), Chicken (C) Type I Antimeningococcus Sera, and of Sera from Human Convalescents (M)

Serum No.	Type	Antibody N removed per ml. serum by Type I meningococci	Serum dilution protecting against Type I meningococci*			
			100,000 M.L.D.	10,000 M.L.D.	1,000 M.L.D.	
H1095	I	mg. 1.15	1/400-1/800	1/350	1/30	
H antitoxin	?	0.56				
Rx (pool)	I	0.24	1/4000-1/8000		1/10-1/20	
RL	I	0.50				
C85 ₁	I	0.55				1/15
M8.2		0.12				
M10.1	I	0.06	1/60-1/70			
M10.2	I	0.10	1/15-1/30			
M14.2		0.05	1/20			
M18.2	?		1/20			

Additional sera tested: M9 failed to protect against 10 M.L.D. in 1/20 dilution; M18. failed to protect against 1000 M.L.D. in $\frac{1}{4}$ dilution.

* 0.5 ml. serum dilution used per mouse.

From (18)

For example, table 6 illustrates the failure to find any correlation between the total agglutinin content and the protective power for mice of Type I antimeningococcal sera (18). In such instances, it may be necessary to obtain an estimate of the amounts of non-protective antibody or to isolate the antigen or antigens associated with protective power from the organisms and establish the correlation of protective power and antibody N by quantitative precipitin determinations with the purified material (cf. II, 11).

Agglutinin estimations involve more manipulations in washing the agglutinated bacteria than do quantitative precipitin determinations. It is necessary to recentrifuge all supernatants and washings to avoid losing small amounts of sediment which do not remain well packed. In some instances, notably with hemolytic streptococci

(16) saponin must be added to eliminate films of bacteria which otherwise form at the surface during washing.

PROCEDURE FOR QUANTITATIVE MICRODETERMINATION OF AGGLUTININS

1. Preparation of bacterial suspensions (14): Cultures of the desired microorganisms are prepared in the usual manner. If the bacteria undergo autolysis readily, 16-24 hour cultures should be used, since accurate results can not be obtained with cultures in which considerable autolysis has occurred. The organisms are centrifuged off from broth (if large volumes of culture are prepared, a Sharples centrifuge is advantageous), suspended in about 1/100 of the original volume of saline and killed by such methods as heating at 60° C. for 45 minutes or addition of formalin to a concentration of 0.5 or 1 per cent and allowing the mixture to stand at 37° C. for 72 hours (14). In the case of both pneumococci (25) and meningococci (18), it has been found convenient to add formalin to the original broth cultures before centrifugation and to heat them at 60° C. for 45 minutes in addition after centrifuging off the organisms and resuspending them in saline. The formalin reduces the possibility of autolysis during centrifugation and the heating destroys the autolytic enzymes; the resultant suspensions are much more stable (26). If organisms are grown on agar, they are carefully washed off the plates with saline, suspended uniformly and killed. The cell suspensions from broth or agar cultures are then washed repeatedly with 0.9 per cent saline or with saline containing 0.025 per cent formalin to avoid disintegration of the organisms if the suspension was formalin-killed, until the supernatants no longer give a Biuret test and nitrogen analyses on 3 to 5 ml. give values within the limit of error of the micro-Kjeldahl method (0.01-0.02 mg. N). Two to seven washings are carried out depending on the ease with which soluble constituents are removed. After each centrifugation the supernatants are drained as completely as possible. The washed cells are finally centrifuged lightly or filtered through a small wad of absorbent cotton to remove large particles and suspended in a volume of saline such that each milliliter of suspension contains 0.15 to 0.60 mg. of nitrogen. Sodium ethyl mercurithiosalicylate is added to a final concentration of 0.01 per cent as a

preservative (cf. appendix) and the suspension kept in a refrigerator. After standing for some time appreciable amounts of nitrogen usually appear in the supernatant and it is necessary to wash suspensions once with saline before use. In the case of Gram positive organisms, suspensions are discarded when smears show that they have become Gram negative. Suspensions of gonococci are taken up in phosphate buffer at pH 5.9 (19).

2. Preparation of stromata (21, 27): Washed packed erythrocytes are laked by pouring into 20 volumes of distilled water or into 10-20 volumes of distilled water saturated with CO_2 . The stromata are centrifuged off, washed 2-3 times with cold 0.2 per cent NaCl and suspended in saline containing 1:10,000 sodium ethyl-mercurithiosalicylate and kept in the refrigerator. Portions are washed once more just before use. Heated stromata are reported to centrifuge more readily and to be slightly less soluble, but their antibody-binding capacity is reduced (21).

3. Preparation of lipid floccules Eagle, (22, 23): 50 grams of dried powdered beef heart are extracted for 15 minutes at $30-37^\circ \text{C}$. with 250 ml. ether with frequent shaking. The mixture is filtered with suction and the residue again extracted with 250 ml. ether. After four extractions the powder is washed on the filter with ether, dried and extracted with 250 ml. absolute ethyl alcohol for 3-5 days at room temperature, with periodic shaking. The alcoholic extract is then filtered and the moist powder washed with absolute alcohol until the combined alcoholic extract and washings measure 250 ml. To this extract cholesterol and corn germ sterol are added each to a final concentration of 0.6 per cent.

This antigen is diluted as needed by rapidly blowing 1.3 volumes of 4 per cent NaCl into one volume of antigen. On dilution with the 4 per cent NaCl the sterols precipitate a suspension of microscopic crystals containing the active factor on their surface. This mixture is kept in the icebox for 2 days to permit better crystal formation and is then centrifuged and the crystals washed twice with a mixture of 1 volume absolute alcohol and 1.3 volumes of 4 per cent NaCl and finally suspended in the desired volume of saline containing preservative.

4. Preparation of specific precipitates (24): Since antigen antibody precipitates formed in the region of excess antigen do not resuspend readily, it is advisable to add a quantity of antigen which

will leave a small amount of antibody in the supernatant. The precipitate is centrifuged off, suspended uniformly and washed with 0.9 per cent saline until the supernatants show no precipitate or only a slight constant turbidity on boiling. The precipitate is suspended in a known volume of saline with addition of preservative and aliquot portions are analyzed for nitrogen. From the amount of antigen used, the volume of washed suspension obtained and its nitrogen content the ratio of antibody N to antigen can be computed, since all of the antigen is contained in the specific precipitate, if the antigen is a single substance and a small amount of antibody has been left in the supernatant.

5. Antisera: Before setting up quantitative agglutinin estimations, antisera should always be freshly centrifuged in the cold for $\frac{1}{2}$ to 1 hour. Any particles of lipid at the top of the tube should be carefully sucked off and the clear serum carefully decanted from any sediment. Control tubes of serum and saline are always set up with each analysis and any blank value obtained is deducted from the total agglutinin N. With weak antisera a precipitate in the serum control tube can be a serious source of error. This can be obviated by using only sera from which any traces of particulate matter have been removed by thorough centrifugation. When using suspensions of *Hemophilus influenzae* B, some strains of meningococci or stromata it is necessary to adjust the pH of the antisera to 6.7 to 7.0 before centrifugation to avoid dissolving some of the suspension N (17, 22). With gonococci, sera are adjusted to pH 5.9 (19).

Where necessary sera may be inactivated at 56° C. After inactivation, however, sera should be recentrifuged in the cold before use to avoid high blank values.

6. Analytical procedure: A number of determinations may be run at one time. From 0.5 to 4.0 ml. of serum, depending on the potency of the serum, are added in duplicate from accurately calibrated pipettes to Wassermann tubes. 2 to 3 ml. of the uniformly mixed bacterial (or other particulate) suspension are added to each sample from an accurately calibrated pipette. Bubbles often tend to form when bacterial suspensions are pipetted; they may be avoided by filling the pipette slowly (cf. appendix). Blanks containing an equal volume of the suspension plus saline in place of serum are set up at the same time, and also a control consisting of

serum plus saline without bacteria. For adding the suspension, a Krogh pipette (28) may be used to advantage but bubbles must be avoided with particular care (17). The contents of the tubes are then thoroughly mixed by a rotary motion imparted by repeatedly drawing the fingertips rapidly and diagonally down the sides of the tubes. The tubes are placed in the water bath at 37° for 2 hours and then in the icebox over night, or the experiment may be conducted entirely at 0° and left for 24 or 48 hours, with occasional mixing. Visible agglutination usually occurs immediately if the serum is fairly strong, and if any agglutinin is present, the serum suspension tubes should appear more turbid than the blanks containing bacteria. If the bacteria flocculate, the contents of the tubes are gently mixed every 15 or 20 minutes as described above while in the water bath to aid in the establishment of equilibrium. After standing overnight the tubes are centrifuged in the refrigerated centrifuge at about 2,000 R.P.M. An additional portion of suspension may be added to each tube if it is believed that an excess of suspension was not present initially and the tubes again mixed and placed in the icebox for an additional 24 or 48 hours. If care is taken not to resuspend the first portion of suspension, it is usually possible to determine whether the second portion was agglutinated. The blanks in some instances have to be run at higher speeds or for longer periods of time, since the unagglutinated organisms are frequently more difficult to centrifuge tightly. The supernatants are completely decanted and the tubes allowed to drain provided the deposit shows no tendency to run down the sides. In many instances, particularly in the case of the blanks, recentrifugation of supernatant is necessary (see below) to avoid loss of bacteria.

If purified antibody solutions are used aliquot portions of the supernatants may be analyzed for nitrogen by the micro-Kjeldahl method and the value obtained subtracted from the total nitrogen of the antibody solution to check on the N uptake of the bacteria.

After draining, the tubes are placed in ice-water. The precipitates are broken up by tapping the tubes, 0.5 ml. of ice-cold saline is added to each tube and the precipitates are uniformly suspended. The tubes are then rinsed down with 2.5 ml. of cold saline as in precipitin determinations and the contents again mixed. The tubes are allowed to stand in ice-water for $\frac{1}{2}$ hour and centrifuged again

in the cold. The supernatants are again decanted, the tubes drained and the precipitates washed a second time in a similar manner, after which the supernatants are decanted and the tubes drained. In instances where large volumes of serum are used, three washings with saline are preferable. The precipitates are then suspended in water and quantitatively transferred to 100 ml. pyrex micro-Kjeldahl flasks with the aid of distilled water, finally with water containing a few drops of normal sodium hydroxide. The micro-Kjeldahl analysis for nitrogen is then carried out in the usual way. It is advisable to pour off the supernatants separately into marked tubes, for it is frequently found that traces of agglutinated or unagglutinated bacteria are present and an additional centrifugation is necessary. After the minute deposit which is thus generally obtained has been freed from the supernatant, the first washing from the main tube corresponding with it may be poured upon it, rinsing down the sides of the tube with a little additional saline. After this is centrifuged and decanted, the second washing from the main tube is added, and after this has been centrifuged the deposits from the main tube and the corresponding tube are transferred to a micro-Kjeldahl flask and analyzed for nitrogen (III, 12). Results are calculated as follows:

Milligrams agglutinin N, for volume of serum used, = N determined — N in bacterial suspension blank.

Agglutinin N \times 6.25 = agglutinin in milligrams protein for the volume used.

The agglutinin N content of a serum in milligrams per milliliter is the maximum value obtained by dividing the agglutinin N found by the number of ml. used. If total agglutinin N is desired the amounts of serum and suspension should be adjusted so that antigen is in excess. Complete removal of antibody is checked by setting up aliquot portions of the supernatants with a second portion of the bacterial suspension. If the value for nitrogen obtained is the same as that in the suspension alone, all of the agglutinin was removed in the first instance, and the number of milligrams N per ml. equals the total agglutinin N content. If a small additional amount of nitrogen is precipitated in the determination on the supernatants it is calculated back to the original volume and added to the first value. Unless the greatest accuracy is desired, analysis of the supernatants need not be completed if, after 48 hours in the cold,

with occasional mixing, the tubes show no greater sedimentation or evidence of agglutination than the controls. Not more than a few hundredths of a milligram of antibody nitrogen is likely to be present in a supernatant showing no visible agglutination (17).

With some suspensions of streptococci, Henriksen and Heidelberger (16) observed that on centrifugation there was a marked tendency of the bacteria to form films on the surface of the fluid and stick to the walls of the tubes. This effect was most marked in the tubes containing only the bacteria and saline and resulted in greater loss of nitrogen from these tubes than in those containing agglutinated suspensions. This tended to make the agglutinin N values too high. The effect could be eliminated by the addition of saponin (16):

"0.2 ml. of 1 per cent saponin solution was added to each of the empty tubes into which the control suspension supernatants were to be poured. This was repeated after each centrifugation, before the next supernatant was poured into the tube. In the case of the supernatants from the agglutinated cells, saponin was added to the tubes only when these were to receive the supernatants from the first, second, and third washings, since it was usually necessary to run a further quantitative absorption on the original serum supernatant. Moreover, losses due to film formation did not occur in the presence of much serum."

Saponin should never be added to the control suspensions or to the tubes containing serum since it causes even greater losses of nitrogen.

To obtain an estimate of the magnitude of losses in washing the bacteria, it is advantageous to combine and analyze the supernatants from the duplicate control tubes containing the bacterial suspension and saline and also to analyze the washings of these tubes for nitrogen. The values found are frequently useful as an indicator of errors in technic, disintegration of suspensions, or excessive losses in washing. With well-prepared bacterial suspensions and careful manipulation, the losses in washing should be below the experimental error of the method ± 0.02 mg. N per analysis.

For measuring the antibody nitrogen content of Wassermann-positive sera with lipid floccules, Eagle used 40 ml. samples of serum and carried out the analyses in 50 ml. centrifuge tubes (22).

With purified antibody solutions the Folin-Ciocalteu phenol reagent may be used to measure the decrease in nitrogen in the

supernatant after removal of antibody by lipid floccules (23). Increasing amounts of suspension are added to several portions of antibody solution and maximum antibody absorption estimated at the point where the nitrogen content of the supernatant reaches a constant minimum value.

QUALITATIVE AND SEMI-QUANTITATIVE METHODS OF MEASURING AGGLUTININS.

These procedures include slide and test tube agglutination tests, the latter being preferred for titrations of agglutinins by dilution and optimal proportions methods, using particulate antigens. They are universally used in immunological studies and are also of great value to the immunochemist for preliminary tests before using the quantitative analytical methods or when these techniques are not applicable.

They are used in the classification of microorganisms, in blood grouping and in numerous diagnostic procedures, and also provide rapid and convenient relative methods for assay of the antibody content of antisera, as shown below.

Slide agglutination: This simple procedure is used for blood grouping, for flocculation tests for syphilis, and for bacterial agglutination. Spot plates, glass microscope slides or thin plates about 4 x 3 cm. may be used. To avoid spreading of the liquid, with the latter, small paraffin circles about 5 mm. in radius are placed on the plates. With infectious materials, tests are more safely performed in Petri dishes (28b). For the actual test a drop of suspension is placed in several circles on the slide and to each a drop of the serum or serum dilution to be tested is added. A circle containing a drop of suspension and one of saline or normal serum serves as a control. For mixing, the glass plate may be gently rotated while resting on the table or a mechanical device may be used. After a suitable interval the degree of agglutination is observed visually or microscopically.

A typical slide agglutination procedure for determining the blood group is briefly as follows: A 4 per cent suspension from packed erythrocytes is prepared in saline. One drop of suspension is added to each of two circles on the glass plate. To the first a drop of known blood group A serum (containing agglutinins for B cells) and

to the other a drop of known blood group B serum (containing agglutinins for A cells) is added. The drops on the plate are mixed and observed for five to ten minutes. Agglutination of the cells is noted. The behavior of cells of various blood groups is shown in the table. Prior to transfusion cross matching is also carried out in addition to the establishment of the blood group of both donor and recipient. This is done by mixing serum of the recipient with cells of the donor and vice versa. If no agglutination occurs in either mixture, the bloods are not incompatible. It is also of interest to use suspensions of known blood group A and B erythrocytes to test for isoagglutinin in the serum of individuals. The table lists the distribution of isoagglutinogens A and B and their isoagglutinins in various blood groups:

Blood Group	Agglutination with		Antigen in cells	Isoagglutinin in serum
	A serum (anti-B)	B serum (anti-A)		
O	—	—	—	anti A and anti B
A	—	+	A	anti B
B	+	—	B	anti A
AB	+	+	AB	—

As is evident from the table, blood from a single individual does not contain corresponding agglutinogen and agglutinin. For further details on blood grouping, and blood group factors other than A and B, see (29).

Test tube agglutination: Test-tube agglutinations are preferred when agglutination requires more than a few minutes and evaporation might preclude the use of the slide technic or when many titrations involving serum dilutions are to be compared. The procedure is conveniently carried out in a volume of 0.2 to 1 ml. in tubes about 75 x 10 mm. For hemagglutination tests 0.2 ml. of a 4 per cent suspension of washed erythrocytes is satisfactory; with bacteria 0.2 or 0.5 ml. of suspensions of turbidity 5 or 6 by McFarland scale (see Appendix) may be employed. Flocculation tests with lipid suspensions may also be performed in this manner (28a).

In carrying out the test decreasing volumes of serum are added to a series of test tubes. The amount of serum in each successive tube may be decreased by one-half, one-third, one-fifth, etc., as desired. Dilutions in twofold steps may readily be made up in the tubes by

adding 0.5 ml. of saline to each tube except the first. 0.5 ml. of the lowest dilution to be tested (undiluted serum, 1:10 serum, etc.) is added to the first and second tubes. The contents of the second tube are mixed, 0.5 ml. withdrawn and added to the third tube, its contents mixed and 0.5 ml. withdrawn and added to the fourth tube, etc. The extra 0.5 ml. from the tube containing the highest serum dilution is discarded. A control tube containing 0.5 ml. of saline is also set up. A separate pipette should be used in making each dilution or the entire length of the pipette may be rinsed with saline after each step. The tubes contain undiluted, 1:2, 1:4, 1:8 etc., dilutions of serum, if undiluted serum was used, or 1:10, 1:20, 1:40, 1:80, etc., with 1:10 serum etc. If necessary additional saline is added to each tube, followed by 0.2 ml. of the erythrocyte suspension or 0.5 ml. of the bacterial suspension. The contents of the tubes are mixed and incubated at 37° C. or at 55° C. for 1 to 4 hours and read. In the case of bacteria they may be placed in the refrigerator overnight. Time and temperature of incubation should be adjusted to give optimal results for each individual system. The degree of agglutination is read by gently tapping the tube and noting the degree of clumping as compared with the control tube. In some instances, readings are facilitated by light centrifugation of the particles, especially when stromata or collodion particles are used in agglutination tests. Results may be graded from — to + + + +. The titer of the serum is the highest dilution of serum giving definite agglutination. The procedure permits comparison of the antibody content of various antisera in relative terms. A serum which agglutinates in 1:80 dilution is usually considered to be four times as potent as one which agglutinates in 1:20 dilution. For maximum accuracy in comparing relative antibody activities, sera should be set up at the same time with the same suspension. Variations in reading endpoints of one tube may occur when tests are repeated on different days. Under optimum conditions a precision of a factor of 2 may be obtained. The technic employed by Hirst and Pickels (38) may also be adapted for measuring agglutination of bacteria and erythrocytes by antibody.

Barrett and Tripp (30) have reported that measurement of the agglutination titer of an antiserum with a given pneumococcal suspension serves as a useful guide in determining the amount of that bacterial suspension to be added for quantitative agglutinin esti-

mation. Table 7 shows the correlation obtained by these authors between the agglutinin titer using two-fold serial dilutions as described above and the total agglutinin nitrogen content of 182 Type I antipneumococcal sera. It is apparent from the table that there is a definite correlation between the two sets of values probably because most of the antibody in type-specific antipneumococcal sera is type-specific anticarbohydrate, although all the antisera

TABLE 7
Correlation between Agglutinin Titer and Antibody-Content of 182 Type I Antipneumococcal Sera

No. of sera tested	Agglutinin titer	Mg. Antibody-Nitrogen per ml. serum		
		Minimal value	Maximal value	Median
22	1:8 or less	0.0	0.3	0.1
11	1:16	0.1	0.5	0.2
15	1:32	0.3	0.8	0.5
25	1:64	0.3	1.6	0.6
23	1:128	0.7	2.1	1.1
30	1:256	1.6	5.4	2.3
21	1:512	2.6	9.8	5.4
35	1:1024	5.9	12.2	7.8

From (30)

Courtesy of Williams and Wilkins Co.

probably contained some antibody to several other antigens, i.e. to the group specific carbohydrate, and to the proteins of pneumococcus.

It must be emphasized that the relationship between antibody nitrogen content and agglutination titer varies with different antigens. It has been pointed out (14), that a Type I antipneumococcal serum containing 1.56 mg. agglutinin N per ml. may agglutinate only to a titer of 1:80 whereas an antiserum to IR pneumococci containing only 0.26 mg. agglutinin N per ml. may agglutinate IR organisms to a titer of 1:800. These variations in the ratio of agglutinin titer to antibody N content in different organisms are probably a manifestation of differences in the combining ratios of the respective antigen-antibody systems and of the amount of each antigen at the bacterial surface. Therefore, in interpreting results obtained using agglutinin titrations, care should be taken to restrict the comparisons to systems involving a single variant of a microorganism.

Certain strains of microorganisms which are spontaneously agglutinated in saline, such as R pneumococci, may be obtained in

uniform suspensions using 0.4 per cent NaCl or less. If agglutinin titrations are carried out at low salt concentration reading of end points with R organisms is greatly facilitated.

Hemagglutination by substances other than antibodies: Certain substances, notably influenza A and B and other viruses (31-33) and the plant hemagglutinins such as concanavalin A (34), ricin, abrin, robin, hurain (35, 36) etc., cause agglutination of erythrocytes. The agglutination test with twofold serial dilutions as described above, may be used as a measure of the amounts of these substances. Estimation of the hemagglutinating activity, per milligram nitrogen or dry weight, of various fractions obtained during chemical manipulation of these substances, or determination of the minimum amount of nitrogen giving definite hemagglutination under a set of standard conditions, may frequently be a very valuable aid in following the course of purification.

For assay of the hemagglutinating potency of fluids containing influenza virus, Salk uses 0.5 ml. of a 0.25 per cent suspension of washed chicken erythrocytes. Tests are read after 1½ to 2 hours, by comparison of the patterns formed on settling. In tubes in which no agglutination occurs, the cells settle as a central, sharply demarcated round disc. Where agglutination is maximal, a pink film covers the entire bottom of the tube as if the clumps adhered to the bottom of the tube at the point of settling. Intermediate reactions usually appear as irregular clumps with a halo of finely aggregated or unagglutinated cells. Any standard system of making readings is satisfactory, however. A hand lens is frequently of assistance. A similar technic is employed in reading Rh agglutination tests (37).

Hirst and Pickels (38) have described a densitometer for measurement of the point of 50 per cent agglutination and Miller and Stanley (32) used the 50 per cent end point determined with the aid of a Klett-Summerson photoelectric colorimeter with a special adapter. Somewhat greater precision is obtained by these methods.

Hemagglutination inhibition tests: If a hemagglutinin, such as those mentioned above, is mixed with its homologous antibody in suitable proportions and a suspension of erythrocytes is then added, hemagglutination does not occur. This property may be used in assaying the relative potency of antisera to these substances by measuring the minimum amount of serum capable of completely inhibiting hemagglutination of a given volume of erythrocyte sus-

pension by a constant amount of hemagglutinin. This type of method has been used in assaying antisera to influenza virus (31, 33) and antibodies to concanavalin A (34). The procedure is carried out as follows:

The minimum amount of a given solution of a hemagglutinin causing agglutination of 0.2 ml. of a 4% suspension of washed erythrocytes (sheep, human, rabbit as desired) is determined. This may be called 1 unit (100% unit). For satisfactory precision in hemagglutination inhibition assays, a solution of hemagglutinin is prepared such that 0.2 ml. contains about 10-20 minimum hemagglutinating units. To a series of tubes (75 x 10 mm.) decreasing amounts of the antiserum to be tested are added, for example: ml. undiluted serum: 0.1, 0.08, 0.06, 0.05, 0.04, 0.03, 0.025, 0.02 ml. The serum should be added in volumes of 0.1 to 0.6 ml. of a suitable dilution. In the foregoing example the first five tubes would contain 0.5, 0.4, 0.3, 0.25, 0.2 ml. of a 1:5 dilution and for the remaining three tubes 0.3, 0.25, 0.2 ml. of a 1:10 dilution are added. Saline is added to each tube to a volume of 0.6 ml. and 0.2 ml. of the hemagglutinin solution is added. The contents of each tube are mixed thoroughly and incubated at 37° C. for 30 minutes. Then 0.2 ml. of 4% suspension of washed erythrocytes in saline is added, the contents of each tube again mixed and incubated at 37° C. for one hour. It is advisable to mix tubes after 30 minutes. Two control tubes are set up simultaneously, one containing 0.8 ml. of saline and the other containing 0.2 ml. of hemagglutinin solution and 0.6 ml. of saline. After the 30 minute incubation, 0.2 ml. of the erythrocyte suspension is added to these tubes which serve, after the final incubation, as controls for absence of hemagglutination and for four plus hemagglutination. The degree of hemagglutination of each tube is recorded and the minimum volume of serum causing complete inhibition of hemagglutination is taken as the endpoint.

If the amount of hemagglutinin solution used in each tube is standardized on a nitrogen basis, the results may be expressed as the amount of hemagglutinin nitrogen neutralized by 1 ml. of serum. For example, if 2 micrograms of hemagglutinin N was added to each tube and 0.02 ml. of serum was the minimum required for complete inhibition of hemagglutination, 1 ml. of serum would neutralize 100 micrograms N.

Alternately, if 20 minimum hemagglutinating doses were used in

the test and 0.02 ml. of serum was taken as the end point, 1 ml. of serum would neutralize 1000 minimum hemagglutinating units.

With very weak antisera, it may be necessary to use smaller amounts than 10-20 units of hemagglutinin for the assays. Thus in the assay of antisera to influenza virus, Hirst employs four 50 per cent units of hemagglutinin and Salk recommends one 100 per cent unit, and twofold serial dilutions of serum.

* The titers obtained and the sensitivity vary with the number of hemagglutinin units used in the test. Selection of a suitable procedure for individual needs, based on preliminary experiments is necessary.

A technic based on inhibition of hemagglutination has also been used in the estimation of blood group A and B substances (39, 40) and may also be used for the estimation of Forssman antigen. Solutions of these substances when added to their respective antisera combine with the antibody and prevent it from agglutinating erythrocytes. The relative amounts of these substances in various solutions may be compared by determining the highest dilution of each solution causing complete inhibition of hemagglutination. Morgan and King (39) assayed solutions for blood group A substance by mixing 0.1 ml. portions of two-fold dilutions of the solution to be tested with 0.1 ml. of a dilution of human serum containing antibodies to A substance (3 to 4 100 per cent hemagglutinating doses). The tubes were allowed to stand at room temperature for 1 hour, 0.1 ml. of a 0.5 per cent suspension of A erythrocytes were added, the tubes shaken, allowed to stand, and read after 2 hours at room temperature. The highest dilution showing complete inhibition of hemagglutination was taken as the end point. This procedure proved very useful in following changes in activity during purification of the blood group substances. Hemolysis inhibition tests may also be used (cf. I, 4).

Agglutination tests with collodion particles coated with antigen: When soluble antigens are available, increased sensitivity over that of the precipitin test may be obtained by using collodion particles. Two technics have been employed; the use of suspensions of particles coated with antigen (9-11) or the addition of collodion particles directly to mixtures of antigen plus antiserum; so-called "collodion fixation" (12).

Preparation of collodion particles (Modified from 9): Col-

Iodion USP (non-flexible, Merck) is poured into approximately 2 liters of distilled water which is being stirred with a glass rod. The collodion separates from the solution and is then washed in three changes of distilled water by decantation. The mass is then dried at 40° C. and a 5 per cent solution in acetone is made by stirring at 40° C. Glass stirring rods should be used throughout and the mixture should not come in contact with metal surfaces at any time. This collodion-in-acetone solution is kept as a stock solution.

When particles are needed, the solution is placed in a 400 ml. beaker which is surrounded by a water jacket at 40° C., and is stirred with a T-shaped glass arm rotated by a small motor at about 1000 r.p.m. while to it is added a mixture of 3 parts distilled water and one part acetone in a fine, continuous stream. For this and all subsequent stages double (4I), or better triple-distilled water should be used. Approximately 30 ml. of water-acetone mixture is required for 75 ml. of original solution; at the end point a heavy gelatinous portion separates from the solution, leaving a clear supernatant which becomes cloudy on addition of excess water-acetone mixture. The cloudy supernate is then decanted into a filter flask containing about 300 ml. of cold distilled water, which becomes densely cloudy as a result of separation of the particles. The gelatinous precipitate may be redissolved in acetone and the process repeated as often as desired, the supernates being added to the filter flask. When enough particles have been produced, the flask is attached to a water-aspirator pump or other source of vacuum until the odor of acetone becomes faint. This takes from 2 to 5 hours, but if this step is omitted the particles will stick together while they are being washed. The suspension is then decanted through a thin cotton filter to remove larger particles of collodion and the filtrate is centrifuged in an angle centrifuge at 3000 r.p.m. for 5 minutes and the supernate decanted. The precipitated particles are resuspended in water and removed from the centrifuge tube. The supernate is centrifuged again for a similar period and the procedure repeated until the supernate becomes relatively clear. All of the particles so obtained are pooled and washed twice more by the same procedure in order to ensure complete elimination of the acetone. The mixture of particles is finally centrifuged more slowly (1500 to 1800 r.p.m.) for about one minute, in order to eliminate the larger particles, and the supernate is poured off and retained as the stock suspension of particles.

Coating of particles with antigen: When the particles are to be coated with antigen, a suspension dense enough to be milky is added to about twice its volume of antigen solution and the mixture is allowed to remain overnight in the refrigerator. The antigen solution should have been previously dialyzed as free as possible from salts; when heavy ions such as calcium, sulfate or phosphate are present in the solution electrodialysis may be desirable. The antigen solution is concentrated in a cellophane bag by exposing to an electric fan for several hours or overnight, until the desired concentration is obtained. With crystalline egg-albumin, solutions of from three to five per cent have been satisfactory (9). If the particles settle while in contact with the antigen, they are resuspended by shaking. They are then washed by the same method of centrifugation given above except that they do not sediment as rapidly. If they are centrifuged too briskly they will stick together. They are resuspended in double (41) or triple distilled water, and the pooled mixture is washed three times in all to insure freedom from antigen in the suspension fluid. After the final washing the pooled particles are taken up in distilled water so that the suspension appears milky, and to this is added a buffer solution of pH 6.0 in the proportion of one part buffer solution to ten of suspension. The buffer solution is prepared by mixing 12.5 ml. of 0.2M sodium acetate and 0.3 ml. of 0.2 M acetic acid and diluting to 250 ml. It tends to stabilize the particles so that the suspension can be kept in the refrigerator for a period of several weeks and portions are removed for dilution with isotonic salt solution (0.9 per cent) when titrations are to be made.

Assay of agglutinin titer: Progressive two fold dilutions of antiserum are made, as described above, so that each tube contains 1 ml. of the serum dilution. To each tube is added one ml. of a suspension of antigen-coated collodion particles diluted in saline solution to a turbidity corresponding to tube 5 of the McFarland scale (see appendix). The tubes are shaken, kept at room temperature for ten minutes and centrifuged, together with a series of controls containing normal serum similarly diluted, at approximately 1200 r.p.m. for four minutes. The tubes are then shaken and the titers determined as the highest dilution of each serum showing definite agglutination of the collodion-particles. If the suspension is stable, the particles will resuspend readily and smoothly

in the tubes containing normal serum. Because the titrations are easily and quickly performed, many sera can be tested in a period of a few hours, which makes it possible to use the same suspension of sensitized particles and identical conditions of time and temperature throughout.

“Collodion fixation” technic (from 12): A suspension of collodion particles of density such that a 1:10 dilution corresponds to number 3 on the McFarland scale (see appendix) is prepared. This suspension is added in 0.1 ml. amounts to a series of tubes containing a constant amount of antigen. To these mixtures, appropriate dilutions of immune serum are added and the volume adjusted to 1 ml. with saline. The tubes may be centrifuged and read after 1 hour at room temperature or may be left overnight in the icebox. Readings are made by comparison with a control tube set up without serum. Parallel controls with normal serum should also be performed.

Difficulties with collodion particle agglutination: Several workers have called attention to sources of error in carrying out these tests. Collodion suspensions tend to aggregate on standing and such aggregated suspensions will agglutinate in control tubes containing normal serum. If this occurs, prepare a fresh suspension. Furthermore, the sensitivity of the test varies with the amount of collodion particles used. With either too light or too dense a collodion suspension very weak titers are obtained (41,42).

Cold hemagglutination: If hemagglutination tests are carried out at low temperatures ($0^{\circ} - 5^{\circ} \text{C.}$), certain sera are found to agglutinate a wide variety of homologous and heterologous erythrocytes (44). The reaction may be reversed by warming to $25-37^{\circ} \text{C.}$, and reagglutination occurs on cooling. These cold agglutinins may be absorbed on erythrocytes or stromata at low temperatures, and eluted at 37°C. In one instance a serum showing cold hemagglutinins to a titer of 1/2560 was found to contain 1.47 mg. N per ml. removable by absorption on stromata at 0° . Electrophoretic patterns on the serum before and after absorption of the cold agglutinins showed a decrease in area of the gamma globulin of 16 per cent after absorption (43, 44). Cold hemagglutinins in low titer are commonly found in normal and animal blood. Increased titers of cold hemagglutinins have been found in a variety of pathological conditions, including atypical pneumonias, trypanosomiasis, hemolytic anemia and paroxysmal cold hemoglobinuria

not associated with syphilis (44). Patients with cold hemagglutinins do not appear to be excessively subject to transfusion reactions if proper cross matching is carried out. A detailed review is given in (44).

In carrying out cold agglutination tests, blood samples are divided in half. One-half of the sample is added to oxalate crystals and the other allowed to clot. Both samples should be kept at 37° C. The tube containing the oxalated plasma is centrifuged, preferably at 37° C., the supernatant discarded and the erythrocytes washed three times at 37° C. with 3 volumes of 0.9 per cent saline, allowing the cells to remain suspended for 15 minutes at 37° during each washing. After the third washing cells should not be auto-agglutinable at 0-4° C. If auto-agglutination occurs further washings are indicated. A 1 or 2 per cent cell suspension is then prepared. Serum is obtained from the clotted blood, by centrifugation, preferably at 37°; all erythrocytes should be removed.

Tests may be carried out by addition of 0.1 ml. of cell suspension to 0.4 ml. of twofold serial dilutions of serum from 1/10 to 1/2560 and to a control tube containing saline. All tubes are placed in ice water and kept in the icebox overnight. The contents of the tubes should be mixed several times during the first few hours.

Hemagglutination is recorded in the usual manner, keeping all tubes in ice water while making readings. Care should be taken to make readings rapidly to avoid warming during the time required for examination. The titer is taken as the highest dilution giving hemagglutination.

In carrying out ordinary hemagglutination tests, precautions should be taken to exclude the possibility of non-specific cold hemagglutination.

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CHAPTER 4

COMPLEMENT AND COMPLEMENT FIXATION

Investigations on complement or alexin have been attended by extraordinary difficulties due largely to its lability and complexity, so that even after more than fifty years of study, knowledge of these elusive protein constituents of serum is still fragmentary. Although complement has long been employed as a useful tool by the immunologist, its chemical nature remained obscure until very recently. The mechanism of the lytic action of complement is also as yet unexplained.

Complement and complement fixation have been reviewed extensively by Osborne (1) and more recent advances have been treated by Pillemer (2). The present chapter will be restricted to fundamentals necessary for an understanding of the action, properties and uses of complement with emphasis on recent investigations and more practical aspects.

The discovery of complement emerged from observations in the 1880's by Buchner (3), von Fodor (4), and Nuttall (5) that blood serum exerts a destructive influence upon bacteria. Nuttall (6) found that this bactericidal power decreased as the serum aged and was rapidly lost on heating at 56° C. Following Pfeiffer's (7) discovery of the lysis of cholera vibrio injected into the peritoneal cavity of immune guinea pigs, Bordet (8) demonstrated bacteriolysis *in vitro* and also showed that the bacteriolytic power of immune serum, when destroyed by heating to 56° C., could be restored by addition of normal serum, establishing that two distinct substances were required for bacteriolysis.

One, termed complement by Bordet and considered identical with Buchner's alexin, was present in normal sera, and could be readily destroyed by heat. The other, a more stable factor termed the sensitizing substance or amboceptor, was an antibody which was produced or at least increased as a result of immunization. Later, it was shown that specific hemolysis was produced by two analogous factors, namely, complement and the antibody which in this instance was termed hemolysin. The ease with which the hemolytic reaction could be observed led to its widespread use for the detection and assay of complement.

As a result of the work of Ehrlich and Morgenroth (9), it was found that the hemolysin or amboceptor could combine with homologous erythrocytes in the presence or absence of complement. Sufficient amounts of hemolysin caused hemagglutination in the absence of complement. Erythrocytes, however, do not take up complement unless hemolysin is present. The union of red cells, antibody and complement leads to hemolysis only if conditions such as temperature or concentration of reagents are suitable. Since fresh normal guinea-pig serum possesses powerful complement activity, it is the usual source for this reagent. Hemolysin is obtained by preparing rabbit antisera to erythrocytes, usually sheep erythrocytes. Complement in these sera is destroyed by heating or aging.

A fundamental property of complement is its capacity to participate in immune phenomena, as for example, its ability to add to certain antigen-antibody combinations. In fresh immune serum or in a mixture of aged or heated immune serum and fresh normal serum containing active complement, the rate and character of immune aggregation is modified due to the incorporation of complement into the antigen-antibody complex. When red blood cells or certain susceptible bacteria are used as antigen, the uptake of complement under suitable conditions leads to lysis of the cells. Thus hemolysis and bacteriolysis may be regarded as special cases of the general property of complement of combining with antigen-antibody complexes. Complement may also combine with previously formed specific precipitates. Addition of a finely divided washed specific precipitate to fresh guinea-pig serum results in fixation of the complement to the immune aggregate. The fixation of complement is readily demonstrable with the aid of sensitized erythrocytes (i.e. erythrocytes plus hemolysin) as test reagent for complement. If complement has been fixed by a specific precipitate, it is no longer available for the lysis of sensitized red cells. This is the basis of the complement fixation test devised by Bordet and Gengou (9a).

The uptake of complement by specific precipitates has recently been demonstrated directly by an increase in weight (10-15). These experiments provided for the first time an absolute, quantitative measure of the complement content of guinea-pig (10-11), human (14) and bovine sera (15). Since complement is not a single substance but a complex of several serum constituents, and since

the extent to which each of these components of complement is fixed by specific precipitates is as yet not clearly established, the weight increase measured by Heidelberger is tentatively ascribed to the "combining components" of complement, whichever these eventually turn out to be. A more detailed treatment of this question will be given after the components of complement have been discussed.

Hemolytic complement activity may be estimated in terms of the smallest amount of fresh serum which will produce complete lysis of a specified portion of sensitized red cells. The unit of complement defined in this way is, of course, arbitrary. It depends mainly on the number of red cells employed, on the amount of sensitizing antibody, on the volume in which the reaction is carried out and on the salt concentration of the system (68). However, other factors to be discussed later also may play a role.

When amounts of complement of less than one unit are used, partial hemolysis results, that is, only a fraction of the red cells added are lysed. Studies of this region of partial hemolysis, have shown that the relation between the amount of complement used and the fraction of cells lysed is not linear, but follows a course described by a S-shaped curve of the type shown in fig. 5a. It is evident from the shape of these curves that complete (100 per cent) hemolysis is approached only gradually, and thus relatively large increases in complement are required to effect lysis of the last 5 to

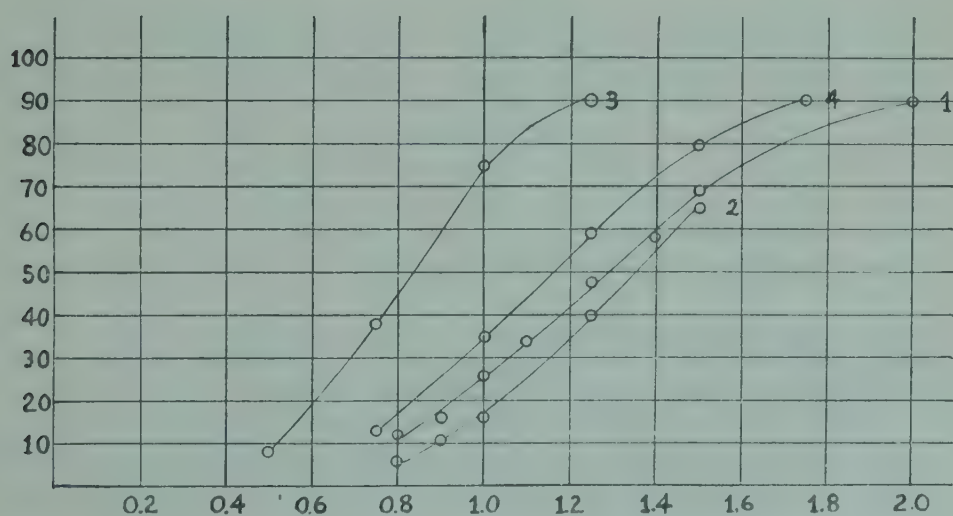


FIG. 5a. Plot of percent hemolysis (y) against ml. (x) of diluted guinea pig serum.
Ordinate % y. Abscissa % x.

10 per cent of cells. In the central region, however, the curve is steep, indicating that here the reaction is sensitive to small changes in the amount of complement employed. The use of complete or 100 per cent lysis as an endpoint, therefore, does not afford as sensitive an index of hemolytic complement activity as an endpoint in the central region of the curve, for example, at the point of 50 per cent lysis (cf. 16-21).

The activity of complement in terms of the 50 per cent unit can be estimated from measurements of the degree of hemolysis with several different amounts of complement so chosen that the determinations fall between about 20 and 80 per cent lysis. Degrees of hemolysis are plotted as ordinates against the quantities of complement used, and the 50 per cent unit is found where the curve of the experimental points intersects the 50 per cent ordinate. The four complements represented in fig. 5a, for example, have the following 50 per cent units: 1.29, 1.35, 0.84, and 1.16. Since the central region of the curve, viz. between 30 and 70 per cent lysis, is almost linear, a good approximation can be based on only two points suitably spaced within this range.

More convenient and accurate methods of measuring complement activity can be derived from the equation of von Krogh (22)

$$x = K \left[\frac{y}{1-y} \right]^{1/n} \quad [1]$$

which describes the course of the hemolysis curve (fig. 5a). In this relation, x represents the amount of complement employed (in ml. of diluted guinea pig serum) and y stands for the degree of lysis (i. e., 100 y equals per cent hemolysis).

The constant K is the 50 per cent unit of complement since at this point $y = 0.5$ and the term $\frac{y}{1-y}$ equals unity and therefore $x = K$.

The constant $1/n$, which determines the shape of the S-curve, has been found in many independent experiments to have a value of $0.2 \pm 10\%$ (68, 69). The von Krogh equation was derived as an adsorption formula, but owes its characteristics largely to the well-known inhomogeneity of red cells with respect to their susceptibility to lysis (70, 71).

It has been noted (16, 68) that the value of $1/n$ deviates markedly

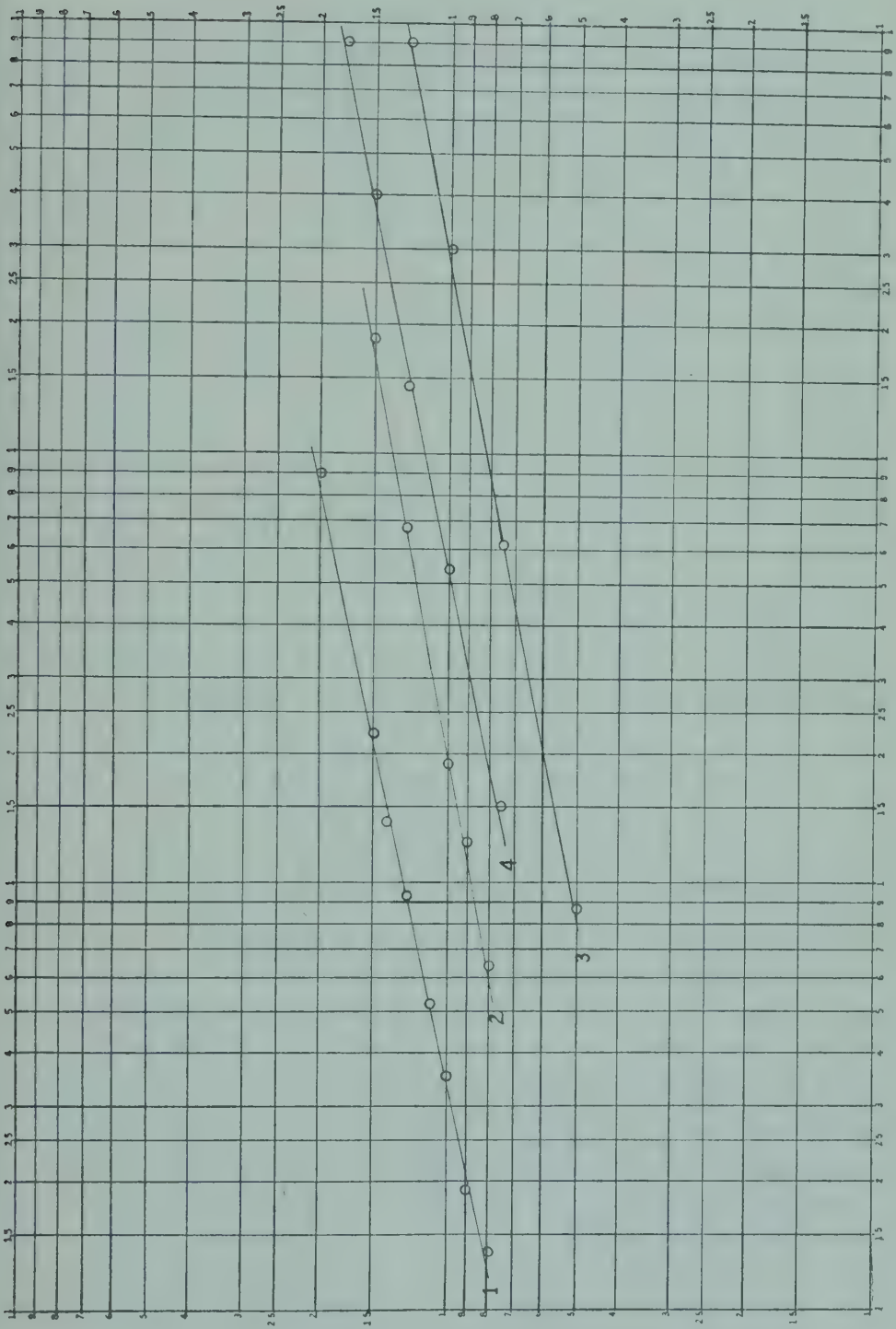


FIG. 5b. Logarithmic plot of x against $y/1-y$. Ordinate = $y/1-y$. Abscissa = $y/1-y$.

from 0.2 when the different amounts of complement distributed in tubes for titration are subjected to incubation at 37° C. prior to addition of sensitized red cells. This can be ascribed to the fact that the loss of complement activity which occurs during incubation is more marked in higher dilutions, so that the different amounts of complement set up for titration do not deteriorate uniformly. As a consequence, the quantities of *active* complement remaining after incubation are no longer related to one another in the proportions in which they were distributed in the tubes, resulting in a value for $1/n$ differing from 0.2.

It follows from equation [1] that every degree of hemolysis bears a determinable relation to that of 50 per cent lysis, in terms of the relative amounts of complement required. The 50 per cent unit of a lot of complement can therefore be derived from a single analysis in the range of partial hemolysis. To avoid cumbersome calculations, Maltaner et al (16-21) have advocated the use of factors for calculating the activity of complement in terms of 50 per cent units from an analysis in the range between 10 and 90 per cent lysis.

These factors are obtained as follows: If the amount of complement required for 50 per cent lysis is assigned the value 1, and if $1/n = 0.2$ is substituted in equation [1], the expression

$$x = \left(\frac{y}{1-y} \right)^{0.2} \quad [2]$$

is obtained which relates x , the amount of complement giving any degree of lysis (y) to 1, the quantity of complement required for 50 per cent lysis. Values of x for the range $y = 0.10$ to $y = 0.90$, calculated from equation [2], are compiled in table 1.

These values for x are the factors used to calculate activity of complement in terms of 50 per cent units from a single analysis in the range of partial lysis. For example, if 1.0 ml of a 1:100 dilution of complement produces 30 per cent lysis (i.e., $y = 0.30$), the activity equals $100 \times 0.844 = 84.4$ units.

Experimental determination of the value of $1/n$ involves the use of equation [1] in logarithmic form:

$$\log x = \log K + \frac{1}{n} \log \left(\frac{y}{1-y} \right) \quad [3]$$

This relation describes a line of intercept $\log K$ and slope $1/n$. A

TABLE I.
Conversion Factors Calculated From the Von Krogh
Equation for $1/n = 0.2$

Degree of lysis	Factor	Degree of lysis	Factor
0.10	0.644	0.55	1.041
0.12	0.671	0.60	1.084
0.14	0.696	0.65	1.132
0.16	0.718	0.70	1.185
0.18	0.738	0.75	1.246
0.20	0.758	0.80	1.320
0.25	0.803	0.82	1.354
0.30	0.844	0.84	1.393
0.35	0.884	0.86	1.438
0.40	0.922	0.88	1.490
0.45	0.961	0.90	1.552
0.50	1.000		

series of accurate, closely spaced dilutions of complement is set up for titration, the results are plotted, and the slope of the resulting line is taken as the value of $1/n$. Plotting is facilitated greatly by the use of logarithmic graph paper. Titrations with four different lots of complement are represented in this manner in fig. 5b. The respective slopes are 0.22, 0.19, 0.19 and 0.21.

The graphical method of utilizing equation [3] can also be applied to direct determination of the 50 per cent unit by evaluation of the intercept, $\log K$. This method is more laborious than the use of conversion factors since at least two points are required for construction of the line, but for the same reason it is more reliable than analyses based on a single point, provided the slope of the line is checked and found to be near 0.2. The values of K , i.e., the 50 per cent units of complements No. 1 to 4 in fig. 5b, as given by the intercepts with the abscissa 1.0, are, respectively, 1.27, 1.36, 0.82 and 1.15, in good agreement with those derived from fig. 5a (see above). The choice of the proper abscissa 1.0 is made by inspection of the degrees of lysis of the experimental points.

Hemolysin and complement: It is well known that hemolytic activity of complement depends on the amount of hemolysin present.

If the quantity of hemolysin in the system is increased, hemolytic activity is enhanced, and a given dose of complement appears more active. Measurements of complement activity must therefore be performed with constant, specific amounts of hemolysin. The relationship between complement and hemolysin is illustrated in the series of complement titrations given in table 2.

TABLE 2
Titration of Complement and Hemolysin

Hemolysin ml. $\times 10^{-5}$	Hemolysin N* microgram	Complement, ml.				
		10×10^{-3} C'N = 0.5 μ g	6.25×10^{-3} C'N = 0.3 μ g	4×10^{-3} C'N = 0.2 μ g	2.5×10^{-3} C'N = 0.13 μ g	1.67×10^{-3} C'N = 0.08 μ g
6	0.03	c	c	c	c	+++ \neq
4	0.02	c	c	c	c	++
3	0.015	c	c	c	+++ \neq	++
2	0.01	c	c	+++ \neq	++	+
1.5	0.008	c	c	+++	+	\neq
1	0.005	+++	++	+	0	0
0.75		++	+	+	0	0
0.5		+	\neq	0	0	0
0		0	0			

Symbols: 0=no hemolysis. \neq =trace, +=slight, ++=moderate, +++=strong, +++ \neq =almost complete, c=complete hemolysis.

* By quantitative analysis of total antibody for sheep stromata (23).

Modified from Table II in ref. 12.

It is apparent that the 100 per cent endpoint (complete hemolysis), that is the unit of complement, equals 6.25×10^{-3} ml. of guinea pig serum when 0.008 microgram of hemolysin N is used, while with 0.015 microgram of hemolysin N the endpoint lies at 4×10^{-3} ml. of complement. If the amount of hemolysin is increased still further a limiting activity is, however, approached. Hence, when such an excess of hemolysin is present the complement unit becomes less dependent on variations of hemolysin dilution. An excess of hemolysin is, therefore, needed to sensitize cells for use in complement titrations. It has recently been shown (72) by quantitative titration that the hemolytic activity of complement at different levels of hemolysin varies as a hyperbolic function.

Speed of immune hemolysis: The length of time required for hemolysis by complement depends on the amounts of hemolysin and complement present, the temperature of incubation and the concentration of the reagents, that is, the final volume to which cells,

hemolysin and complement are diluted. At 37° C., the usual temperature of incubation, in the presence of a moderate excess of hemolysin, as recommended below, and at a final red cell concentration of about 100 million cells per 0.8 ml., hemolysis terminates in about 30 to 45 min., no matter what the degree of hemolysis. With tubes containing more than one 100 per cent unit of complement, of course, complete lysis will occur much sooner. If the final volume of the system is increased hemolysis will proceed more slowly.

If a reaction time of only 15 min. is used, as preferred by some investigators, the exact temperature of the incubation bath, the room temperature and the speed with which the tubes warm up when first placed in the waterbath become factors of importance. They may be ignored if a longer period of incubation is allowed.

While mammalian complement lyses most rapidly at about 37° C., frog complement has been shown to be almost as active at 15-16° C. as at 37° C., and carp complement is active even at 0° C. (74).

Effect of total volume of the lytic system on the 50 per cent titer of complement: The measurements tabulated below were carried out with an incubation period of 45 min. One hour of incubation did not result in an appreciably higher titer in the case of the 7.5 ml. volume.

Total volume of lytic system, <i>ml.</i>	2.0	3.0	5.0	7.5
Titer, 37.0°C., 45 min.	272	211	148	108

From (68). (Dose of sensitized red cells = 500 million)

Effect of salt concentration: Recent quantitative measurements (68), listed below, have shown that the 50 per cent titer of complement varies markedly with the salt concentration of the hemolytic system.

Molarity of NaCl + buffer	0.145	0.151	0.155	0.162	0.168	0.173	0.179
Titer	143	126	118	96	83	75	64

(veronal buffer, pH 7.4).

Effect of temperature of incubation (68): As shown below,

the 50 per cent titer of complement varies somewhat with the temperature of incubation:

Temperature, °C.	39.0	38.0	37.0	35.3	32.4
Titer after 45 min.	133	139	144	147	147

Effect of pH (68): Only slight variations of 50 per cent titer have been observed between the limits of pH 6.9 and 7.6:

pH	6.9	7.1	7.4	7.6
Titer	158	155	162	162

(Veronal-NaCl buffers, 0.151 *M*, used).

Effect of Mg^{++} : It has been shown recently (68) that Mg^{++} is essential for the hemolytic action of complement. The hemolytic system as ordinarily constituted does not contain sufficient Mg^{++} for optimal hemolytic activity, so that a marked enhancement can be obtained by addition of extra Mg^{++} . This observation makes it possible to ascribe the enhancing action of certain tissue or culture fluids on complement activity to their contribution of Mg^{++} . On the other hand, substances like citrate or pyrophosphate which bind Mg^{++} are anticomplementary when added to the usual hemolytic system which contains only a small quantity of Mg^{++} . This type of anticomplementary effect can be overcome by addition of extra Mg^{++} .

PROCEDURE FOR THE TITRATION OF COMPLEMENT

Preparation of reagents: (Note: keep all reagents, including saline for dilution, ice-cold, unless otherwise noted.)

1. Suspension of sheep erythrocytes: 10 ml. of sheep blood, defibrinated by gently shaking in a vessel containing glass beads, or citrated with 2 ml. of 4 per cent sodium citrate solution is diluted with 30 to 40 ml. of 0.9 per cent sodium chloride solution (saline) and centrifuged for 5-10 minutes at about 1500 r.p.m. in a 50 ml. graduated centrifuge tube. The supernatant fluid is drawn off and the red blood cells are suspended in about 40 ml. of saline. After 5-10 minutes' centrifugation, the supernatant fluid is sucked off again. The washing process is repeated twice more in the same

manner. The final supernatant should be entirely colorless. If it is tinged with hemoglobin, as may occur in five to seven days after the blood is drawn, the cells are too fragile and fresh blood should be obtained. As much of the packed sediment of red cells as needed for the day's work is diluted with 19 volumes of saline to yield a "5% cell suspension," which is then filtered through a small wad of absorbent cotton to remove any blood clots. Unused packed cell sediment may be kept in the refrigerator for future work but should be washed again at least twice immediately before use. The washings serve to remove free hemoglobin released during storage, but if the cell suspension continues to yield colored supernatants after repeated washings, the cells are too fragile and should be discarded.

It has been shown recently that sheep blood drawn aseptically into an equal volume of Alsever's solution* (68,73) and stored in the cold can be used for complement titrations over a period of several months. The cells stored in this medium become constant in their susceptibility to lysis by complement and antibody after four days' storage and remain at the same level of susceptibility for at least ten weeks. A portion of the mixture is removed aseptically each day, centrifuged and washed as described above.

In precise, quantitative studies of complement activity such as those in (68) it has been found preferable to use a veronal-NaCl buffer† of pH 7.3 to 7.4 instead of isotonic saline. This buffer is used for dilution of all reagents and for the washing of the cells.

Since the 5% cell suspension is likely to vary somewhat in respect to its red cell content, it is preferable in precise studies to standardize by direct cell count, or better, by colorimetric determination of hemoglobin.

A cell count of the 5% suspension is made in a hemocytometer using a 1:40 subdilution, and the 5% suspension is adjusted to 1 million cells per cu. mm. either by addition of the required amount of saline or by centrifuging and resuspending the cells in the proper volume calculated from the count.

For colorimetric standardization lyse 1.00 ml. of the 5% cell sus-

* 2.05 gm. glucose, 0.8 gm. sodium citrate, 0.42 gm. sodium chloride per 100 ml. Adjust to pH 6.1 with citric acid. Sterilize by passage through a bacteriological, sintered glass filter.

† 85.0 gm. NaCl, 5.75 gm. 5,5-diethyl barbituric acid, 3.75 gm. sodium 5,5-diethyl barbiturate. Dissolve the acid in 500 ml. hot water, add to the solution of the other components, cool and make up to 2000 ml. with water. Each day dilute accurately 1 part up to 5 with water; the pH of the diluted buffer should be 7.3 to 7.4.

pension by addition of 14.0 ml. of distilled water. Measure the optical density, D , of the resulting hemoglobin solution at 5500 Å in a colorimeter. In a Coleman Universal Spectrophotometer (No. 11) an average value of $D_{5500} = 0.56$ was found equivalent to a cell concentration of 1 million per cu. mm. using a 13 mm. cell and reading against water as a blank. The optical density of the hemoglobin solution was found to be linear up to a concentration of about 1.1 million cells per cu. mm., but deviates somewhat above that value. Therefore, a calibration curve should be established for use in the required adjustment of the 5% suspension to a concentration which would yield $D_{5500} = 0.56$. The standard value of the optical density corresponding to 1 million cells per cu. mm. should be redetermined if a different colorimeter or other cuvettes are employed.

The wavelength of 5500 Å was chosen because it represents an absorption maximum for oxyhemoglobin in measurements with the Coleman Spectrophotometer. Actually, oxyhemoglobin has two absorption peaks in this region but these appear merged with the Coleman instrument. It has been noted recently that a partial conversion to methemoglobin occurs in the course of a complement titration and this leads to a slight decrease of optical density since at 5500 Å methemoglobin absorbs less light than oxyhemoglobin. However, at 5200 Å oxy- and methemoglobin exhibit the same light absorption, so that no difficulty would arise if readings were made at that wavelength.

Nevertheless, the use of 5500 Å has been continued for the present in our laboratory because a change of wavelength requires readjustment of the total volume of the hemolytic system which results in a change of complement activity, and also because the small errors due to methemoglobin formation tend to cancel when the degree of lysis is calculated by division of one value of optical density by another.

2. Sensitization of cells: Hemolysin may be obtained by immunization of rabbits with washed sheep erythrocytes or stromata, but satisfactory antisera may be purchased. The adjusted cell suspension is sensitized by addition of an equal volume of the proper hemolysin dilution. This dilution is determined by titration of each new lot of hemolysin, and is checked at least every 3 to 6 months by the technique described under 4. The well-mixed hemo-

lysin dilution is poured slowly into the cell suspension with constant agitation, the mixture incubated at 37° C. for 10 minutes and kept in ice-water thereafter. Sensitized cells cannot be kept from one day to the next, so that a fresh lot should be prepared each day.

3. Complement titration: (Note: dilutions of complement are good for one day only).

a) **100 per cent lytic unit:** Set up 0.2 ml. portions of sensitized cells (100 million red cells) in small test tubes (12 x 75 mm.) with 0.075, 0.100, 0.125, 0.150 and 0.200 ml. of a 1:40 dilution of fresh guinea pig serum or of serum stored in dry ice. (Lyophilized complement, available commercially, may also be used, but is likely to be somewhat less active, necessitating the use of a 1:20 dilution. If complement is preserved frozen in dry ice for long periods dilute sodium bicarbonate solution may have to be added to neutralize the acidity developed as the result of CO₂ uptake. This can be avoided by storage of complement in sealed glass ampoules.) Dilute the contents of each tube with saline to equalize the volumes. A final volume of 0.8 ml. is usually employed. Incubate in a water-bath at 37-38° C. for 30 minutes, with occasional mixing to prevent the cells from settling. The cells may be delivered with 1 ml. pipettes graduated in 1/100 ml., but the delivery of the complement dilutions requires 0.2 ml. pipettes with 1/1000 ml. sub-divisions. Scrupulously clean glassware should be used (use of chromate-sulfuric acid cleaning mixture is recommended) so that no drops remain on the walls of the tubes after delivery of the reagents. The addition of saline as the last reagent serves to rinse down traces of guinea-pig serum adhering to the wall of the test tube. It is also imperative to touch the tip of the pipette to the vessel both after setting to the mark and after delivery so that no drops remain on the outside of the pipette near the tip.

The degree of lysis of each tube is estimated and recorded as 0, +, ++, +++ and c (complete). With fresh or frozen complement, complete (100%) lysis usually occurs between 0.1 and 0.2 ml. of 1:40 dilution, corresponding to a titer of 400 to 200 units per ml. The value in units is obtained by dividing the dilution factor (i.e. 40) by the volume which produced complete lysis (i.e. 0.1 to 0.2 ml.).

b) **Determination of the hemolysis curve:**

In the technic described by Wadsworth, Maltaner and Maltaner

(16-21) constant portions of sensitized cells are set up with varying amounts of diluted complement. After incubation the degree of lysis is read by visual comparison with a set of artificial lytic standards. This method has the disadvantage that small volumes of fluid are used, and that visual comparison may introduce subjective errors.

An alternative method (22a) utilizing colorimetric determination of hemoglobin from lysed cells permits more precise estimation of the degree of hemolysis. Since the Coleman Spectrophotometer requires at least five to six ml. of solution for optical analysis, five times as many cells are used as in the 100 per cent endpoint titration, i.e. 1.0 ml. instead of 0.2 ml. When this quantity of cells is lysed, the resulting hemoglobin solution is made to 7.5 ml. because this furnishes a suitable optical density, i.e. 0.56, at a wavelength of 5500 Å. In the earlier description of the method (22a) the lytic reaction was run in a total volume of 4.0 ml. and 3.5 ml. of saline was added *after* lysis to make the required 7.5 ml. This was done in order to maintain constant the ratio, i.e. five-fold, between the number of cells in the 100 per cent and 50 per cent titrations, as well as the volumes namely, 0.8 and 4.0 ml., respectively, so that the titers observed would be comparable. In (68) the reaction volume was given as 5.0 ml. and 2.5 ml. of saline was added after incubation. Recently, a reaction volume of 7.5 ml. has been adopted in order to avoid the addition of saline after incubation, but in this volume a reaction time of one hour is used. It should be recalled that the hemolytic activity of complement depends on the reaction volume, so that titers obtained at different final dilutions are not directed comparable.

The sensitivity of this procedure, however, necessitates certain additional precautions. All glassware should be cleaned with dichromate-sulfuric acid cleaning mixture. Soap-cleaned tubes are definitely unsatisfactory. The following sequence of addition of reagents, differing from that of the 100 per cent method, is recommended because it facilitates requisite mixing and hence uniform distribution of the complement: 1) 1.0 ml. sensitized cells, 2) the amount of saline required to make the desired total volume, 3) 1.0 ml. portions of complement diluted accurately with calibrated pipettes and volumetric flasks 1:110, 1:120, 1:130, 1:150, 1:180, 1:220, added with precision from accurate pipettes, held to the wall of the tube near the surface of the liquid. The reaction is carried out in wide-mouth

50 ml. centrifuge tubes which permit mixing of cells by rotary motion during addition of complement as this is essential for accuracy. The tubes are stoppered or capped to prevent evaporation of water during incubation.

During hot weather it is advisable to set up the reaction mixtures in a bath of cold water to retard the lytic reaction until all tubes are prepared. The entire set is then placed in the waterbath at 37°C . for 45 minutes or one hour with occasionally mixing to keep the cells in suspension. As a further precaution, it is suggested that transfers and mixing operations of the complement dilutions be kept to a minimum.

After incubation add saline to all tubes, if needed, mix, centrifuge, decant each supernatant and measure the optical density at 5500\AA reading against the supernatant of a blank tube of 1.0 ml. of sensitized cells treated like the others except for the substitution of saline for complement. The degree of hemolysis is calculated by dividing the observed optical density by that of a completely lysed mixture.

c) 50 per cent unit: (cf. p.103): To obtain the 50 per cent unit, there is, of course, no need to determine the entire hemolysis curve. Set up two or three analyses in the partial hemolysis range, preferably one below and another above the 50 per cent point. Plot x against $y/1-y$ on logarithmic graph paper and locate the 50 per cent unit where the line through the points intersects $y/1-y = 1$. Alternatively, a plot of x against y on linear paper will yield a good approximation of the 50 per cent unit by linear interpolation. The former is preferable since it permits analyses falling between 10 and 90 per cent hemolysis to be used and since analytical errors may readily be detected by the deviation of the slope from $1/n = 0.2$.

With the aid of the conversion factors in table 1 a single set of duplicate analyses anywhere within the range of ten and ninety per cent hemolysis, or better, between twenty and eighty per cent, will suffice for calculation of the fifty per cent unit. When the reaction volume is 7.5 ml. and no extra Mg^{++} is added to the system, the fifty per cent titer of pooled, fresh or frozen guinea-pig serum is usually about 100 units per ml., so that 1.0 ml. of a 1:100 dilution would yield a suitable analysis. In a reaction volume of 5.0 ml. without extra Mg^{++} , titers are usually near 150 units. Addition of 100 micrograms of Mg^{++} per tube generally increases the titer to about twice these values.

The need for daily titration of complement can be avoided by storage of small portions sealed in Pyrex glass ampoules and frozen in solid CO_2 . Under these conditions the hemolytic activity remains constant for at least several weeks and titrations carried out on different days with portions of the same lot of complement from different ampoules yield the same titers. Therefore, the titer of an entire lot of complement can be determined by titration of the contents of a single ampoule.

4. Titration of hemolysin. The procedure is carried out by setting up 0.1 ml. of two-fold serial dilutions of hemolysin, starting at a 1:100 dilution, with 0.1 ml. portions of unsensitized cells adjusted to proper concentration and 0.2 ml. of 1:20 guinea-pig complement in a total volume of 0.8 ml. Incubate 30 min. at 37-38° C. The highest dilution of hemolysin giving complete lysis is taken as one unit. Use 4 units for sensitization. For example, if the highest dilution giving complete lysis is 1:1600, a 1:400 dilution of hemolysin is mixed with an equal volume of erythrocyte suspension.

It is not advisable to use sera whose hemolytic titer is less than 1:800. Nor should the amounts of hemolysin be large enough to cause agglutination of the erythrocytes. The dilution of hemolysin containing four units should not cause hemo-agglutination in the cold over a period of at least 24 hours.

QUANTITATIVE DETERMINATION OF COMBINING N OF COMPLEMENT ($\text{C}' \text{N}$)

The capacity of complement to combine with specific precipitates affords a means of measuring the serum N associated with the combining components. The method, originated by Heidelberger (10-14), involves determination of the difference in N content of specific precipitates formed in the presence and absence of active complement. Since the actual mass of complement combining components is quite small in contrast to its activity in hemolytic units, it is necessary to use at least two milliliters of active complement for a determination. Numerous previous attempts were unsuccessful due to failure to recognize this.

Thus far, quantitative determinations of $\text{C}' \text{N}$ have been carried out on guinea-pig (11), human (14) and bovine (15) sera. In all three species, the $\text{C}' \text{N}$ per ml. of serum was found to depend on the

amount of complement used for analysis, being progressively smaller when larger amounts of complement were employed for analysis, despite complete removal of complement as determined by absence of hemolytic activity in the supernatant. Fig. 6 shows this apparent solubility effect in the case of guinea-pig C'. By extrapolation to zero volume of C' it was estimated that guinea-pig serum contains about 0.04 to 0.06 mg. C' N per ml. (11), while human serum gave values ranging from 0.03 to 0.05 mg. C' N per ml. (14). Similar results were obtained in the case of bovine sera (15).

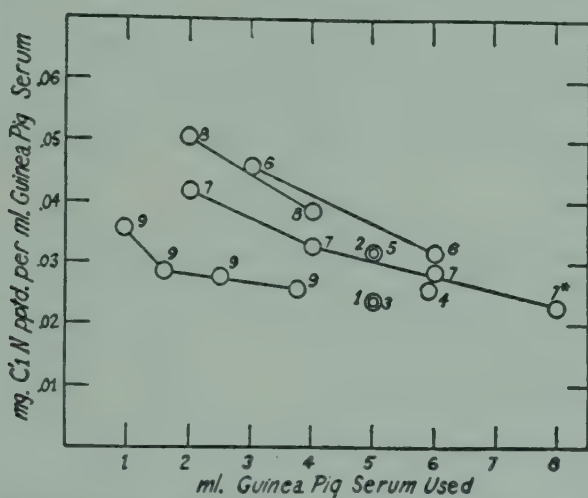


FIG. 6. Amount of complement combining component nitrogen (C'1 N) precipitated as a function of the volume of guinea pig serum used.

The numbers at each point refer to the experiment.

7* = combined serum and S III blank supernatants.

From (11)

The assumption that the N carried down by specific precipitates from active sera constitutes complement protein is supported by several observations. As stated above, hemolytic complement activity is removed by absorption with specific precipitates, that is, after removal of the specific precipitate the supernatant no longer lyses sensitized sheep erythrocytes. When very small amounts of washed specific precipitate are added to a large volume of complement, partial removal of hemolytic activity, as well as of combining N, result. With varying amounts of specific precipitate, uptake of nitrogen by the specific precipitate and disappearance of hemolytic activity from the supernatant run roughly parallel (13).

The fact that no N was taken up from complement inactivated

for 50 min. at 56° C. also supports this assumption. Furthermore, with pneumococcus specific polysaccharide and the corresponding horse antibody, which does not fix complement, as judged by hemolytic activity, no N uptake was observed (11).

Measurement of complement in terms of weight units permits calculations concerning the molecular relationships in immune hemolysis, as summarized in table 3. The number of hemolysin molecules necessary for lysis of a single red cell is so small that only 0.01 to 0.3 per cent of the cell surface would be covered, depending, of course, on the spatial arrangement of the antibody molecules on the cell surface. While these figures are only approximate, they show that sensitization need not involve the entire red cell surface and are therefore in accord with Abramson's "key spot" theory (26). These calculations are discussed in detail in (12) from which table 3 has been taken. Unfortunately, an error in the

TABLE 3

Proportions of Hemolysin, Complement, and Red Cells in Hemolysis

Experiment No.....	1	2	3
C'N per ml. of guinea pig serum, micrograms.....	50	50	50
Fraction of ml. for complete hemolysis of 0.2 ml. sensitized cells.....	0.004	0.004	0.005
C'N necessary for complete hemolysis of sensitized cells, micrograms.....	0.20	0.20	0.25
C' globulin necessary for hemolysis, micrograms	1.26	1.26	1.58
Molecules* of C' necessary for hemolysis ..	5.1×10^{12}	5.1×10^{12}	6.4×10^{12}
Hemolysin N combined per 0.2 ml. of sensitized cell suspension, micrograms.....	0.03	0.03	0.03
Hemolysin globulin used for sensitization, micrograms..	0.19	0.19	0.19
Molecules† of hemolysin used for sensitization.....	1.2×10^{11}	1.2×10^{11}	1.2×10^{11}
Number of sheep red cells in 0.2 ml. sensitized suspension.....	3×10^8	2×10^8	2×10^8
Molecules of C' available for hemolysis of single red cell ..	17,000	26,000	32,000
Molecules of hemolysin combined with single red cell in sensitization and hemolysis.....	400	600	600

* The molecular weight of the combining component of complement is taken as 150,000 (23).

† The molecular weight of rabbit hemolysin is taken as 900,000 (24).

Modified from (12).

surface area of the red cell appeared in that paper. The correct formula is $12/5 \pi A^2$ where A is one-half the cell diameter, and the correct value for the area is $51 \times 10^{-8} \text{ cm}^2$. Furthermore, calculations in (12) were based on an assumed molecular weight of 158,000 for rabbit hemolysin, but the correct value is 900,000 (24). The figures given in table 3 are corrected accordingly.

PROCEDURE FOR QUANTITATIVE ESTIMATION OF COMPLEMENT COMBINING N

The procedure may be illustrated with the aid of the protocol of a typical experiment shown in table 4. Neutralized, centrifuged guinea-pig serum (C') was used. One-half was inactivated at 56°C . for 50 min., instead of 30 min., since it was found (11) that the standard 30 min. period did not always suffice to reduce to a minimum the amount of nitrogen taken up by specific precipitates from inactivated complement (iC'). Both the fresh guinea-pig serum and the inactivated serum were centrifuged in the cold for several hours before use. Diluted, neutralized and inactivated antipneumococcus Type III rabbit serum containing 1.0 mg. antibody N per ml. of dilution* and a solution of homologous specific polysaccharide containing 0.04 mg. per ml. were mixed in equal proportions. The concentrations of antibody and antigen were chosen to leave antibody in excess so that a finely divided precipitate would be formed which could be washed more efficiently than the gelatinous discs obtained in the equivalence zone. Triplicate sets of analyses were performed with active complement (C'), heat-inactivated complement (iC') and with saline as diluent of the immune system. In addition, blanks of C' and iC' were set up with immune serum alone, and with polysaccharide alone. A blank on the immune serum alone was also included. C' and iC' were pipetted into the tubes first, followed by the antiserum. After mixing, the polysaccharide solution was added, the contents of each tube were mixed by twirling (I, 2) and kept at room temperature for 1 hour, or longer if aggregation did not occur within one hour in the tubes containing active complement.

The tubes were then centrifuged in the cold, the precipitates washed three times, and analyzed for N as described for the quantitative precipitin reaction (I, 2) (27). All supernatants were again

* Sera of high antibody content (3 to 7 mg. antibody N per ml.) were used so that analyses could be made at dilutions which were not anticomplementary.

centrifuged as in the quantitative agglutinin procedure (I, 3) (28) to insure the greatest possible accuracy, since the experiment involved measurement of a small difference between two quantities of specific precipitate.

Hemolytic activity in the supernatants from the tubes which had contained active complement was computed from the results on the largest non-anticomplementary volume which could be employed. Complete removal of hemolytic activity was obtained with quantities of specific precipitate about five times as large as the amount of C' N present, as in the experiment shown in table 4.

TABLE 4
Quantitative Estimation of Combining N of complement

No. of tubes.....	1	1	2	1	1	3	3	3
C', ml.....	4.0	4.0						5.0
iC', ml.....				3.0	3.0		5.0	
Serum dilution, ml.....	0.8		1.0	0.6		1.0	1.0	1.0
S III dilution, ml.....		0.8			0.6	1.0	1.0	1.0
N precipitated, mg....	0.012	0.016	0	0.018	0.020	0.584 0.594 0.586	0.634 0.624 0.636	0.740 0.738 0.736
Mean	0.014			0.019		0.588	0.632	0.728
Subtraction of blank.....						0	0.032*	0.018†
Specific N pptd., mg.....						0.588	0.600	0.720
Subtraction of iC' series value.....								0.600
C' N pptd., mg.....								0.12

Hemolytic units left in C' series supernatants, 10 per 5 ml. C' taken.

* $0.019 \times 5/3$.

† $0.014 \times 5/4$.

From (11).

For calculation, the nitrogen obtained in the iC' blank tubes (columns 5 and 6, table 4) was averaged and subtracted from the specific precipitate N in the iC' series (column 8) while the average of the C' blanks (columns 2 and 3) was deducted from the specific precipitate C'N (column 9). Finally, the resulting net values were subtracted (0.720 minus 0.600) yielding the C'N value of 0.12 mg. This is equivalent to 0.024 mg. of C'N per ml. of guinea-pig serum since 5 ml. of serum were used in the analysis. (Cf. fig. 6, experiment No. 1).

COMPONENTS OF COMPLEMENT

Complement is not a single serum constituent but a complex of several substances. At present, four distinct factors, $C'1$, 2, 3, 4 are recognized. The first and second components ($C'1$ and $C'2$) were discovered as early as 1907 by Ferrata (29) who separated guinea-pig complement by dialysis against water into an insoluble and a soluble fraction which were subsequently termed midpiece and endpiece, respectively. The insoluble fraction, when dissolved in physiological saline was devoid of hemolytic activity. The soluble fraction was also found to be inactive, but when the two were combined, activity was restored. It was soon discovered that guinea-pig complement could be split into these two components by a variety of procedures leading to precipitation of eu-globulin, such as by dilution with several volumes of water acidified to about pH 5 or by dialysis against very dilute buffer at about pH 5 to 6.

When complement is treated with cobra venom (30) or yeast (31) or zymosan (32), an insoluble carbohydrate from yeast, its activity is destroyed because a factor other than $C'1$ or $C'2$ is inactivated. This so-called third component of complement, $C'3$, is relatively heat-stable in contrast to $C'1$ and $C'2$ which are very sensitive to heat. Still another thermostable factor, fourth component, or $C'4$, is recognized by the fact that hemolysis can no longer occur after it is destroyed by treatment with ammonia (33) or primary amines (34).

When complement is fractionated by dialysis or by precipitation of the euglobulin, $C'1$ and $C'2$ are the components which are separated. $C'3$ and $C'4$ are distributed in both fractions. Most of the $C'3$ activity remains with $C'1$ in the insoluble fraction while the bulk of $C'4$ is found with $C'2$ in the supernatant.

Within a few minutes after heating at 56° C. complement loses activity because of the destruction of $C'1$ and $C'2$. $C'3$ and $C'4$ are more resistant but if heating is continued for 30 to 40 minutes, most of the $C'3$ and $C'4$ activity may be lost as well.

The evidence for the individuality of the four components may be summarized as follows (cf. fig. 7):

1. The soluble (E) and insoluble (M) fractions obtained on dialysis or by dilution with water, are inactive alone, but activity is restored by reconstituting the mixture.

2. Complement treated with yeast or zymosan (Z) and ammonia-treated complement (N) are inactive when tested separately, but the combination of Z plus N is active.

3. Complement heated at 56°C . for 20 min. (H) alone is inactive but in combination with Z or N hemolysis is obtained. Hence, heated serum contains those components which are destroyed by zymosan and ammonia, namely C'_3 and C'_4 .

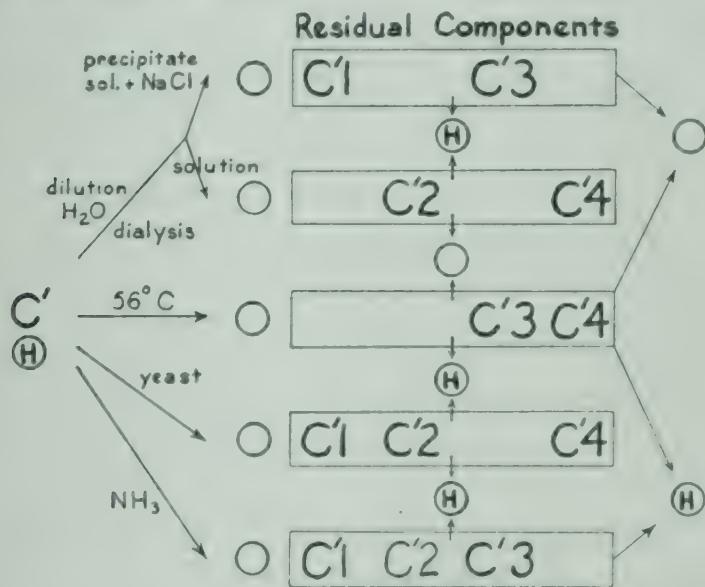


FIG. 7. Interrelations of Complement and its Components. Combinations giving hemolysis are denoted by an H.

4. Heated serum in combination with M or E alone is inactive. Hence, heated serum does not contain C'_1 and C'_2 . It also follows that the components destroyed by zymosan or ammonia, namely C'_3 and C'_4 , are distinct from C'_1 and C'_2 , the factors which are separated when complement is split into M and E.

The insoluble (M) and soluble (E) fractions, zymosan-treated complement (Z) and ammonia-treated complement (N) can be used as reagents in testing for those components which they lack. For example, if an unknown solution possessing no activity when used alone activates zymosan-treated complement (Z), it obviously furnishes the C'_3 which is lacking in Z. Thus Z can serve as reagent for the detection of C'_3 . Similarly, N is a reagent for C'_4 , M can be used to detect C'_2 , and E will demonstrate the presence of C'_1 .

In this fashion, it can be shown that M contains C'_1 , C'_3 and a little C'_4 , E contains C'_2 , some C'_3 and much C'_4 ; Z contains C'_1 , C'_2 ,

and 4, and N contains C'1, 2 and 3. Since most of the C'3 appears in M, while most of C'4 is found in E, M and E have been represented in fig. 7 to contain C'1, 3 and C'2, 4, respectively. The composition of the complement components in various fractions is given in table 5 (36).

TABLE 5

*Average Composition of Whole and Fractionated Complement, and of Specifically Inactivated Complement.**

	Whole C' units/ml.	C'1 units/ml.	C'2 units/ml.	C'3 units/ml.	C'4 units/ml.
G.P. C'	350	2300	450	370	6000
G.P. M, prep'd by dialysis		350	0	120	100
G.P. M, prep'd by dilution		not done	0	100	not done
G.P. E prep'd by dialysis		0	140	60	3500
G.P. E prep'd by dilution		0	220	60	2100
G.P. Z		1000	260	0	2000
G.P. N		1200	300	100	0
G.P. H†		0	0	210	500
Hu. C'	100	3700	170	250	4000
Hu. M prep'd by dialysis		1000	0	90	350
Hu. M prep'd by dilution		1000	0	40	130
Hu. E prep'd by dialysis		0	110	35	2400
Hu. E prep'd by dilution		0	115	45	2400
Hu. Z		2000	100	0	2000
Hu. N		2000	not done	not done	0
Hu. H†		0	0	not done	600

* Compiled from data in (36).

† Heated at 56° C. for 20 min.

As shown by Hegedus and Greiner (35) titrations of the four components in whole C' and C' fractions can be carried out with the aid of the reagents M, E, Z, N and H. The reasonable assumption is made that the titer of whole C' or a mixture of components is a function of the component present in lowest titer, the so-called limiting component. To determine the titer of a component in an unknown, it is merely necessary to add a reagent which does not contain the component to be titrated (as shown by inactivity when used alone) but which supplies an excess of the other three components so that the component in question becomes the limiting component in the mixture of unknown and reagent. The titer of this mixture is taken as the titer of the component sought in accordance with Hegedus and Greiner's assumption. A further essential requirement, sometimes overlooked, is that the reagent should not be anticomplementary in the dose employed, that is, it should not depress or inhibit the activity of whole C'.

A reagent, R₁, for the titration of C'₁ should furnish an excess, that is, more than one unit each of C'₂, C'₃ and C'₄ in the dose employed. The reagent for C'₂, termed R₂, should contain C'₁, 3 and 4. Similarly, R₃ should supply C'₁, 2 and 4, and R₄ should have the composition C'₁, 2 and 3.

R₁ is prepared by combination of E which contains C'₂ and C'₄ and some C'₃, with heated serum (H) which supplies additional C'₃, 4 (cf. table 5). M contains C'₁ and C'₃, but only little C'₄ so that R₂ can be prepared by combination of M and H. Since Z contains C'₁, 2 and 4, and N has C'₁, 2 and 3 in adequate amounts, they can function as R₃ and R₄ respectively (36).

The adequacy of each reagent with respect to its component content is established by testing every reagent with every other one, as follows: 1) R₁ and R₂, 2) R₁ and R₃, 3) R₁ and R₄, 4) R₂ and R₃, 5) R₂ and R₄, and 6) R₃ and R₄. All of these mixtures should be hemolytically active, proving that each reagent actually contains the component it is designed to have. In addition, it is necessary to show that each reagent is inactive alone and that it is not anticomplementary (36).

Although Hegedus and Greiner (35) enunciated the general principle, their materials failed to meet all the requirements outlined above. They used E as a reagent for C'₁, but it has been shown (36) that E does not contain enough C'₃ to be used without H. Similarly, they employed M as the reagent for C'₂, but again heated serum is required to furnish extra C'₃ and C'₄. In the more recent work of Ecker *et al* (37) M and E were not reinforced with H. In addition, these authors did not ensure an excess of components in the reagent thus making interpretation of results difficult.

Another assumption made by Hegedus and Greiner (35) is that the components of complement in the sera of different species are mutually substitutive, that is, a given component from one species could function if the lacking components were supplied by the serum from another species. While the effectiveness of substitution was at first denied in the case of human and guinea-pig C' (37), the recent work of Bier and collaborators (36) showed that the four components of human and guinea-pig serum are mutually substitutive although the exchange may not always be accomplished easily. This conclusion was also reached (37a) by the former workers.

In (36), the four components were titrated in whole human and

guinea-pig sera, in "midpiece" and "endpiece," in the specifically inactivated complements and in heated serum. The required reagents were prepared by standard methods but conditions were carefully established so that the requirements outlined above were met. Results are summarized in table 5. It was found that C'_3 is usually present in lowest titer in guinea-pig complement but that the titer of C'_2 is not much higher. C'_3 therefore is the limiting component of guinea-pig C' . The titer of human C' is limited by C'_2 and the titer of C'_3 is somewhat greater than that of C'_2 . C'_1 titers in guinea-pig and human C' , as well as those of C'_4 , are about the same.

Hegedus and Greiner (35) studied the components of cow, dog, horse, pig, rabbit, rat, sheep as well as guinea-pig and human complement. They found some species such as the cow, horse and sheep to be lacking in C'_2 . Sheep complement lacked C'_4 as well.

PROCEDURE FOR TITRATION OF COMPLEMENT COMPONENTS

Preparation of reagents: A. Midpiece (M) and endpiece (E).

Fractionation of human and guinea-pig complement may be carried out by dilution or dialysis following Ferrata (29), Liefmann (40) and later workers (37, 41). While the CO_2 dilution method (40) may be used for fractionating guinea-pig serum, it is not satisfactory for human serum.

1. Dilution method: To 10 volumes of chilled $M/200$ KH_2PO_4 solution, 1 volume of chilled guinea-pig (g.p.) or human (hu) serum is added slowly, with constant mixing in the cold. After 20 to 30 min. at $0^\circ C$., the precipitate is centrifuged off in the cold, separated as completely as possible from the supernatant and, in the case of guinea-pig C' , washed with phosphate solution of pH 5.4 and ionic strength 0.02. The precipitate from human C' need not be washed since human serum contains much less C'_2 than does guinea-pig serum. The precipitate, or M, is dissolved in saline and brought to pH 6.5 to 7 by cautious addition of a freshly prepared 0.1 N $NaHCO_3$ solution. It is made up to 5 times the original serum volume for further dilution as needed. The original supernatant, or E, is made isotonic with 10 per cent $NaCl$ and adjusted to about pH 7.5 with 0.1 N $NaHCO_3$ as soon as possible since C'_2 is unstable below pH 6.5 (42, 43). The final dilution is 1:12.

2. Dialysis method (37) Fifteen ml. of human serum in cellophane tubing is dialyzed in the cold for 24 hours on a mechanical stirrer (III, 33) against 4 liters of phosphate buffer of ionic strength 0.02 and pH 5.4. For larger amounts of serum more time should be allowed and the phosphate buffer should be changed once or twice. Bier and coworkers (36) shortened the dialysis period to 6 hrs. when 5 ml. portions of serum were used, to avoid deterioration of components unstable at acid pH. This was accomplished by increasing the dialyzing surface by placing a Pyrex test tube inside the cellophane bag to force the serum into the resulting annular space, by frequent mixing and by changing the outside buffer solution every 2 hours.

After dialysis the contents of the cellophane bag are centrifuged in the cold, the supernatant is separated and neutralized by careful addition of 0.2 ml. of 0.1 *N* NaOH per ml. of supernatant. The solution is then made isotonic with 10 per cent NaCl.

The precipitate (M) is washed twice with cold phosphate buffer of ionic strength 0.02 and pH 5.4 and dissolved in saline. The final dilutions are 1:5 for M and 1:10 for E.

The phosphate buffer for dialysis and washing is prepared by adding 153 ml. of 0.5 *M* KH_2PO_4 solution and 1.73 ml. *N* KOH to sufficient distilled water to make 4 liters of solution.

B. Complement lacking C'3: The third component is inactivated by treatment with "zymosan," an insoluble carbohydrate prepared from yeast (32). The method of treatment given here is that recommended in (34, 37).

Boil 100 mg. of zymosan in 10 ml. of saline for one-half hour. Centrifuge, discard the supernatant and suspend the sediment in 100 ml. of saline. This stock suspension which contains 1 mg. per ml. is stored in the cold. It should be mixed well before use.

Ecker (37) recommends the use of 1.35 mg. of zymosan for inactivation of 1 ml. of human serum. 1.35 ml. of the stock suspension is pipetted into a test tube, centrifuged and the supernatant discarded. 1 ml. of human serum is added to the sediment taking care to obtain a smooth suspension. The mixture is incubated at 37° C. for 1 hour with occasional mixing to keep the zymosan in suspension. Centrifuge, pour off the inactivated serum and dilute with 4 ml. of saline to obtain an approximately 1:5 dilution with respect to original serum volume.

Guinea-pig serum requires about 15 times as much zymosan for inactivation of C'3 (36) as human serum. The optimal quantity of zymosan for human as well as guinea-pig serum should be determined by experiment, since different preparations of zymosan may vary in potency. It may also be necessary to allow $1\frac{1}{2}$ or 2 hours instead of 1 hour of incubation. Choice of the amount of reagent and time of inactivation should be based on actual tests for completeness of C'3 inactivation with minimal destruction of the other components.

C. Destruction of C'4: To 1 ml. of serum is added 0.25 ml. of 0.15 *N* NH₄OH. Incubate for $1\frac{1}{2}$ hours and neutralize with 0.25 ml. of 0.15 *N* HCl. Add saline to a final dilution of 1:5 with respect to original serum volume.

D. Heat inactivated serum: Human and guinea-pig sera are heated at 56°C. for 20 min. This is sufficient to inactivate C'1 and C'2 and is less destructive for C'3 and C'4 than 30 minutes' heating. Add saline to a dilution of 1:5.

Titration: The titers of individual components are estimated by serial dilution as in an ordinary complement titration except that a constant amount of reagent (R₁, R₂, R₃, or R₄) for the component in question is added to all the tubes. The amount of reagent to be employed must be determined in a preliminary experiment. The amount chosen must be inactive when tested with sensitized sheep cells, and it must not be anticomplementary. Anticomplementary properties are ascertained by testing the effect of the reagent on $\frac{1}{2}$, $\frac{3}{4}$ 1, and $1\frac{1}{2}$ 100 per cent units of whole C'. If one unit, or even $1\frac{1}{2}$ units, fail to lyse, the reagent is anticomplementary and should be used at a lower concentration. In the case of R₁ and R₄ an enhancing effect is usually observed since these reagents supply components which are present in low titer in human and guinea-pig sera. R₂ is the reagent which most commonly exhibits anticomplementary effects especially when the mid-piece, which is part of this reagent, is old and has been allowed to become too alkaline. Each reagent should supply an excess of those components it is designed to furnish.

The sequence of tests in a typical experiment is summarized below:

1. Test M, E, Z, N, and H alone for complete inactivation, using from 0.05 to 0.4 ml. of a 1:5 dilution in two-fold steps.

2. Check the anti-complementary properties of M, E, Z, N and H. Set up the largest quantity which proved completely inactive in test No. 1 with amounts of a 1:40 guinea-pig complement dilution ranging from 0.05 ml. to 0.3 ml. In testing for anticomplementary action against human C', use a dilution of 1:10 or 1:20 of the latter.

Use not more than one-half the least anticomplementary dose of a reagent for the subsequent tests.

Tests No. 1 and No. 2 thus define the *upper limits* for the use of each reagent.

3. Titrate H against Z as follows:

ml. 1:5 or 1:10 dilution of H

ml. 1:5 or 1:10 dilution of Z				
	0.05	0.08	0.13	0.20
	0.05			
	0.08			
	0.13			
	0.20			

This titration should determine the lower limits of activity of H and Z.

A similar series of titrations of H against N is done next, but since the titer of C'₄ in H is quite high, a dilution of 1:20 or 1:40 of H should be used.

These tests will show how much H should be used in reagents R₁ and R₂. It has been found best to keep E and H, and M and H apart and to add them separately to the tests requiring R₁ and R₂.

4. A series of titrations of Z against N, using Z at a dilution of 1:10 and N at a dilution of 1:5, as in 3 above, should yield information regarding the *lower limits* for Z and N as reagents R₃ and R₄, respectively.

5. M and E are titrated against one another in the presence of the optimal amount of H, as determined in test No. 3. M can usually

be run at a dilution of 1:20 while E is ordinarily used at 1:10. This experiment should furnish the *lower limits* for M and E.

The dilutions indicated here are given for general guidance but may require upward or downward revision depending on the results of the titrations.

The tests, thus far, serve to establish the composition of R₁ (E + H) with respect to its content of C'₂, 3, 4, as well as that of R₂ (M + H) with respect to C'₁, 3, 4. It remains to be determined whether Z and N contain sufficient C'₁ and C'₂ to serve as R₃ and R₄, respectively. This is done by testing Z and N with R₁ and R₂, which are reagents for C'₁ and C'₂, respectively. These reagents are then used in amounts which furnish enough of the desired components and which are not anticomplementary or lytic. If any reagent does not fulfill these requirements, it should be discarded.

Titration proper are run as described for whole complement above, except that the determined amount of the desired reagent is added to the tubes containing serial dilutions of the component to be assayed.

CHEMISTRY OF THE COMPLEMENT COMPONENTS

Pillemer, Ecker, Oncley and Cohn (25) applied the salting-out methods developed by Cohn and his collaborators (38) to the fractionation of guinea-pig complement. C'₁ activity was entirely contained in the fraction of serum protein insoluble in 1.39 *M* (NH₄)₂SO₄, C'₃ was the only component present in the fraction soluble in 1.39 *M* (NH₄)₂SO₄, but insoluble in 2.0 *M* (NH₄)₂SO₄, and C'₂ and C'₄ were soluble in 2.0 *M* but insoluble in 2.5 *M* (NH₄)₂SO₄.

Preliminary to fractionation the complement containing serum was freed from fat by centrifuging in the cold. C'₁ was then precipitated by dialysis in the cold against phosphate buffer of pH 5.2 and ionic strength 0.02. The precipitate was redissolved and brought to 1.22 *M* (NH₄)₂SO₄, centrifuged, and the precipitate redialyzed against the phosphate buffer. This purified fraction retained full C'₁ activity and was homogeneous in the ultracentrifuge and 98 per cent homogeneous by electrophoresis. The yield of material represented 0.6 per cent of the total serum protein. Chemical and physical properties of purified C'₁ are given in table 6.

Eighty to eighty-five per cent of the C'₂ and C'₄ components

were contained in the material soluble in 2.0 but insoluble in 2.2 *M* $(\text{NH}_4)_2\text{SO}_4$. On dialysis against distilled water the two components precipitated as a green transparent viscous sediment which was washed with distilled water and dissolved in 0.9 per cent NaCl.

TABLE 6
Properties of Purified Components of Guinea Pig Complement

	Euglobulin	Muco-Euglobulin
Complement-components present	Mid-piece	End-piece and 4th component
Mobility in phosphate buffer, at pH 7.7, 0.2 ionic strength	-2.9×10^{-5}	-4.2×10^{-5}
Sedimentation-constant 1%, 20°, W.	-6.4×10^{-13}	—
Protein-nitrogen, per cent.	16.3	14.2
Carbohydrate, per cent.	2.7	10.3
Phosphorus, per cent.	0.1	0.1
Optical rotation, $[\alpha]_d^{25^\circ}$	-28.7	-192.6
Apparent isoelectric point.	5.2-5.4	6.3-6.4
Fraction of total complement-activity, per cent.	100	85
Fraction of total serum-protein, per cent*	0.6	0.18
Heat stability of complement, activity (destroyed in 30 mins. at tabulated temperature), °C.	50	50† 66‡

* The three complement-components together then comprise 0.78 per cent of the total serum-protein.

† For end-piece activity.

‡ For 4th component activity.

Data from (25).

The $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis were repeated several times. This "muco-euglobulin" contained 85 per cent of both components C'2 and C'4. Its properties are given in table 6.

A method for the separation and purification of C'1 from human serum has been described by Pillemer et al (39). Human C'1 was characterized as a euglobulin with an electrophoretic mobility of 2.9×10^{-5} in veronal buffer of pH 7.8 and ionic strength 0.1. The material showed two components in the ultracentrifuge. The principal component, comprising 70 per cent of the material, had a sedimentation constant of 6.9 when converted to water at 20°C. The remaining 30 per cent of the preparation sedimented more rapidly.

COMPLEMENT FIXATION

By virtue of its capacity to combine with many antigen-antibody complexes, complement is widely used as an indicator for immunological reactions. In this manner, immune reactions can be detected even when other visible manifestations of antigen-antibody combination such as precipitation or agglutination are absent. A distinct advantage of complement fixation lies in the fact that either soluble or insoluble antigens may be employed. The complement fixation test depends on two properties of complement: 1) the binding or fixing of complement by antigen-antibody aggregates, and 2) the lysis of sensitized erythrocytes. As previously stated, hemolysis may be regarded as a special sequence of the binding of complement by antigen and antibody.

The test itself is performed in two steps. First, the serum to be tested for antibody, a specified dose of complement, and a standard amount of antigen are allowed to react at a given temperature. Sensitized sheep cells are then added and the mixture is incubated at 37°C., usually for 30 minutes. If the serum contains antibody to the antigen used, the complement is bound or fixed when combination of antigen with antibody occurs and is therefore no longer available to lyse sensitized red cells. Thus, failure to obtain lysis denotes a positive reaction, while complete hemolysis indicates a negative result, meaning that the serum did not contain antibody to the antigen employed.

The reaction may also be used to detect antigen, using a serum known to contain antibody. The principle of complement fixation may be represented by the following equations:

Antigen + complement \rightarrow (no fixation) + sensitized cells \rightarrow lysis

Antibody + complement \rightarrow (no fixation) + sensitized cells \rightarrow lysis

Antigen + antibody + complement \rightarrow (fixation) + sensitized cells \rightarrow no lysis

In this manner an antibody-antigen reaction is detected by the fixation of complement, which is made visible by the erythrocyte hemolysin indicator system.

The amounts and concentrations of all reagents must be properly controlled since the test depends upon removal of a limited amount

of complement by the first of two immune systems. The most important of these factors are erythrocyte, complement, and hemolysin concentrations.

Most complement fixation procedures employ two 100 per cent or four 50 per cent units of complement. Since the latter unit may be determined more accurately, its use yields more reproducible results. The test may be made more sensitive by using less complement, but a safe margin above one 100 per cent unit must be allowed to eliminate false positive reactions due to slight anti-complementary effects and to reduce the effects of slight errors in measurement. When $1\frac{1}{2}$ 100 per cent units are used, adequate controls must be run to exclude false positive tests. Some workers prefer $2\frac{1}{2}$ 100 per cent units (cf. Kolmer test, ref. 44).

Sensitivity may also be increased by using larger amounts of hemolysin (12) for, as shown in table 1, the more hemolysin used, the less complement is required for lysis of a given number of cells.

The procedure employed by Eagle (45) is quite different. Instead of determining the complement titer, this author employs a fixed volume of complement and adjusts the hemolytic activity of the system by varying the dose of hemolysin. Thus, the titer of hemolysin rather than that of complement is determined in this procedure.

Since complement can be fixed or rendered inactive by a variety of agents other than immune aggregates, a complement fixation test must be accompanied by certain controls which serve to demonstrate the specific immune character of the fixation. For example, certain bacteria and yeast absorb the third component of complement, thus rendering it inactive without the participation of specific antibody. It is also well known that sera contaminated by bacterial growth frequently inactivate complement in the absence of antigen. When sera or antigens inactivate complement alone (i.e., non-specifically) they are said to be anticomplementary. Consequently, it must be shown in every fixation reaction that neither the serum nor the antigen alone, in the dose employed, affect the activity of complement. This places an upper limit on the amount of serum and antigen which must be determined by experiment.

Thus, the amount of antiserum which may be used with safety in complement fixation is more closely restricted than in precipitin or agglutination tests. Although the antiserum is heated to destroy its complement, the use of too large an amount of serum may exert

an enhancing or inhibiting action on the complement. Either effect is, of course, undesirable. Some investigators prefer to determine the complement unit in the presence of antiserum when a number of antigens are titrated, and to determine the complement unit in the presence of antigen for titrating antisera. The latter procedure is needed with antigens containing Mg^{++} , but may be avoided by addition of optimal Mg^{++} to the system. With the 100 million cell hemolytic system the upper limit for antiserum is about 0.02 to 0.04 ml. More antiserum is tolerated in the case of methods such as that of Eagle (45) in which a fixed excess of complement is employed, and variation of hemolysin serves to adjust the hemolytic activity.

The quantity of antigen is also restricted by anticomplementary limitations. The anticomplementary properties of certain particulate antigens such as stromata, are decreased in the presence of small amounts of inactivated normal human serum. In addition, in some immune systems, too much antigen may weaken the degree of the fixation. Thus the Wassermann reaction (18) and the pneumococcus type-specific polysaccharide systems (46) are sensitive to excess antigen. On the other hand, the reaction between aqueous extracts of tubercle bacilli and serum from tuberculous humans is not inhibited by excess antigen (17). The optimal amount of antigen in any given system must be found by experiment.

The temperature of fixation is also an important factor. In complement fixation tests for syphilis, stronger reactions are observed at 0°C. than 37°C. (47). Fixation tests at 0°C. are usually run for a period of 4 to 24 hours while at 37°C., 1½ to 2 hours represents the upper limit since deterioration of complement is quite rapid at 37°C. With the pneumococcus type-specific carbohydrates (46) two to three times as much complement is fixed in 24 hours at 3-6° as in 1½ hours at 37°C.

The velocity of complement fixation is quite high since, in the reaction between pneumococcus polysaccharide and rabbit antisera (48), it has been shown to take place in three seconds or less, values similar to those found for antigen-antibody combination (49). More intense complement fixation reactions have, however, been obtained after longer time intervals. In the case of particulate antigens, such as bacterial suspensions, reactions are likely to be less rapid than with soluble antigens, but can be accelerated by continuous stirring.

The question of the fixation of the four components has not yet been entirely settled. In recent studies Pillemer, Seifter and Ecker (41) concluded that C'1, C'2, and C'4 combine with specific precipitates but that C'3 is not fixed. Heidelberger, Bier and Mayer (50) also found that C'4 as well as C'1 is fixed completely by antigen-antibody precipitates. The same authors also found that C'2 is fixed, while the uptake of C'3 varied and was not as extensive as that of the other components (51).

TABLE 7

Species Differences in Fixation of Guinea Pig Complement

Species of antibody	Encapsulated pneumococci	Capsular polysaccharide of pneumococcus	Pneumococcus protein	Pneumococcus C-substance	Polysaccharide of H. influenzae
Horse	—	—	+	—	—
Man	—				
Mouse	—				
Cat	—				
Dog	—				
Goat	—				
Rabbit	+	+	+	—	+
Guinea pig	+				
Rat	+				
Sheep	+				
Cow		+			

Modified from (52) and (54).

+ = fixation of complement

— = no fixation

All antigen-antibody systems do not, however, fix guinea-pig complement (cf. table 7). Although some horse antibody systems fix complement, horse antisera to the pneumococcus type-specific capsular polysaccharides do not (52). Similarly, rabbit anticarbohydrate damaged by acid (53) does not bind complement. Four out of six human antisera to the pneumococcal type specific polysaccharides tested (14) fixed human complement in their reaction with specific polysaccharide. Horsfall and Goodner (54) have, however, reported that guinea-pig complement is not fixed by human

sera reacting with encapsulated pneumococci (cf. table 7). In this connection, it should be recognized that concentrations of antibody sufficient to cause agglutination of bacteria may be too low to bind complement in certain systems.

The strength of an antiserum is usually estimated by serial dilution tests with a constant amount of both complement and antigen. Thus it may be found, for example, that all dilutions of serum down to 1:80 give + + + + fixation, while the next successive higher dilutions give + +, + and finally negative tests. When it is desired to test the strength of an antigen, the quantity of antiserum is kept constant while the antigen is diluted progressively. The amounts of antigen present in various fractions obtained during purification may be estimated in this way.

Greater precision of titration may, however, be achieved by the technique of Maltaner (16-21) in which the degree of fixation is measured quantitatively in terms of per cent hemolysis, instead of by the usual crude method in which results are recorded as +, + +, + + + or + + + +.

In general, the method, as used by Maltaner et al. in routine tests for syphilis (19), involves the use of a quotient $(IS + A)/IS$ where $(IS + A)$ is the activity of the complement expressed in 50 per cent units after incubation with immune serum (IS) plus antigen (A), and IS represents the activity after incubation with immune serum alone. After a preliminary titration to determine the 50 per cent unit of the complement, tests are set up with one unit of complement and a fixed quantity of immune serum, and with 3, 6, and 12 units of complement and immune serum plus three doses of antigen so adjusted as to give the maximum reaction in at least one combination with sera of low, moderate and marked activity, respectively. If the immune serum is of moderate potency, the test with 3 units may show no lysis while the 6 unit test may be in the partial range of hemolysis. In case of a strong antiserum both 3 and 6 unit tests may exhibit no lysis but the 12 unit tube may be partially lysed. The degree of hemolysis in the partly hemolyzed tube is read and, using conversion factors such as those in table 1 the amount of complement is found which would have given 50 per cent lysis. For example, if the six unit tube shows 30 per cent hemolysis, $6.0/0.844 = 7.1$ units would have given 50 per cent hemolysis. The degree of hemolysis in the 1 unit tube incubated

with serum alone is also read and converted by application with the proper factor into the amount of complement which would have given 50 per cent lysis. For example, 1 unit of complement in the presence of immune serum may produce 20 per cent lysis. Then $1.0/0.758 = 1.32$ units would have given 50 per cent lysis. The ratio of the amount of complement required for 50 per cent lysis in the presence of immune serum and antigen to that required for 50 per cent lysis in the presence of serum alone, is considered by Wadsworth et al. (19) to be an index of the potency of the immune serum. In this case the ratio would be $7.1/1.32 = 5.4$. Quotients greater than unity are considered evidence of an immune reaction. Titers determined by this method may, however, be affected by the so-called fixability of the complement. Rice (46) points out that variations due to this factor may be overcome by including titrations with a standard serum so that the titer may be expressed in terms of the quotient: $\frac{\text{test (IS + A)}}{\text{test IS}} / \frac{\text{standard (IS + A)}}{\text{standard IS}}$. If the test sera as well as the standard sera are neither hemolytic nor anticomplementary, the quotient may be simplified to the form: $\text{test (IS + A)}/\text{standard (IS + A)}$.

The relations between the amounts of complement fixed and of antibody and antigen in the Wassermann reaction have been extensively studied by Wadsworth and Maltaner (18). They were found to be linear when conditions were adjusted to maximal sensitivity. Linear relationships between serum and complement have also been obtained by Rice with gonococcal sera and antigen (57). The same author also demonstrated linearity between the amounts of rabbit antipneumococcus antibody and complement when maximally reactive quantities of antigen were employed (46). Similar relations have also been observed with tuberculous immune serum and antigen (17).

The results obtained by Heidelberger, Weil and Treffers (12) indicate that a given weight of antibody can fix a weight of complement of about the same order of magnitude. Thus, 0.12, 0.2 and 0.4 micrograms of antibody N fixed 0.2, 0.3 and 0.5 micrograms of C'N, respectively. By direct measurement of the weight of complement carried down by specific precipitates in the presence of excess complement, it could be shown that antibody combined with as much as 72 per cent of its own weight of complement (13).

PROCEDURE FOR COMPLEMENT FIXATION

The method of complement fixation to be described here is one of general applicability. The unit of complement is established by the 50 per cent method cf. p. 112, but the strength of an antiserum is determined by serial dilution and results are expressed in terms of —, +, ++, +++ and ++++ fixation, corresponding to complete, varying degrees of partial lysis and no hemolysis, respectively.

The use of complement distributed in sealed ampoules and stored in solid CO₂ is recommended since it eliminates the elaborate daily titration described in (22a). If desired, the 100 per cent titration method may be substituted when the accuracy does not warrant the more elaborate 50 per cent procedure. In this case the complement is titrated as described above (p. 110), using a 1:40 dilution of guinea pig serum. If, for example, complete lysis occurs at 0.125 ml., 1.25 ml. is diluted to 40 ml. and 0.2 ml. are used for the fixation test, an amount equivalent to two 100 per cent units.

For the complement fixation test 0.2 ml. of a dilution of test serum, inactivated 0.5 hrs. at 56° C. and absorbed with sheep erythrocytes, previous to dilution, is mixed with 0.2 ml. of a guinea pig complement dilution containing four 50% or two 100% units of complement. 0.2 ml. of antigen is then added, the contents of the tubes are mixed and either incubated 40 minutes at 37° C., or 15-20 hrs. at 0°-5° C. After incubation, 0.2 ml. of sensitized red cell suspension is added and the tubes are incubated with occasional mixing for 30 minutes at 37° C. to effect hemolysis. To facilitate readings, the tubes may be placed in the icebox overnight to permit the unlysed red cells to settle. The results are recorded as + + + +, + + +, ++, +, — fixation (equivalent to 0%, 25%, 50%, 75%, 100% of the cells lysed).

Notes

- A. All glassware should be cleaned with sulfuric acid-dichromate cleaning solution. Soap-cleaned glassware is unsatisfactory.
- B. All reagents should be stored in an ice-water bath during use. Complement dilutions and sensitized cells are good only for one day.
- C. Complement in high dilution is labile. Mixing and transfers should be kept to a minimum.
- D. Some workers prefer to titrate complement under conditions analogous to those of the fixation test by incubating the comple-

ment dilution before addition of sensitized cells. If this is done, 0.1 ml. of 1:10 diluted inactivated human serum should be added to each tube to protect the complement from the extensive deterioration which ensues when complement is incubated in the absence of sufficient protein. Titrations carried out without preliminary incubation in the presence or absence of added serum, or with preliminary incubation in the presence of serum, yield practically the same titer. Complement allowed to stand overnight at 0° C. with or without added serum before addition of sensitized cells, also shows about the same titer as in immediate titration. Titration without preliminary incubation and without the addition of serum is therefore satisfactory for incubation at 37° C. as well as in the refrigerator.

E. When high precision is desired, the complement dilution and the sensitized sheep cells should be measured with accurate pipettes.

F. An antiserum dilution of 1/10 is recommended but higher or lower dilutions may be required depending on the system under investigation. In certain instances, positive reactions may occur only over a narrow range of dilution, negative tests being observed with too much as well as too little serum. The lower limit of serum dilution is, of course, determined by the anticomplementary properties of the serum. A given dilution may be used if at least twice the quantity employed is not anticomplementary. The same rule applies to antigen dilutions, where too much antigen may also cause inhibition. Such is the case in the Wassermann reaction for syphilis.

G. Sera which have become contaminated with microorganisms are usually anticomplementary. If stored in the cold under sterile conditions and in the presence of 1:10,000 merthiolate serum samples may be kept fit for complement fixation tests over long periods. Sera which are to be transported should be contained in rubber-capped tubes since cork stoppers may give rise to anticomplementary action.

H. Saline used for dilution should be stored under sterile conditions since contamination with microorganisms may render it anti-complementary. This is especially important when buffered saline is employed. Alternatively, a concentrated stock of salt solution may be kept without sterile precautions and portions diluted to isotonicity each day.

HEMOLYSIS AND BACTERIOLYSIS

In serological studies of erythrocytes and certain bacteria lytic tests with antibody and complement are often more sensitive than agglutination, and titrations of antibody activity by means of lysis are sometimes capable of somewhat greater precision than are assays using agglutination titers. Immune hemolysis has proven particularly useful in studies on heterogenetic antigens of the Forssman type. Substances of this kind are found in the organs or erythrocytes of animals that are widely separated in the zoological system, and even in bacteria. They are characterized by the ability to produce hemolysins for sheep erythrocytes when injected into rabbits and their existence in guinea pig organs was first demonstrated in this manner by Forssman (58). For example, the serum of a rabbit injected with guinea pig kidney can lyse sheep erythrocytes in the presence of complement. Schiff and Adelsberger (59) demonstrated that a substance related to the Forssman antigen exists in human erythrocytes of blood groups A and AB since injection of these cells into rabbits resulted in the production of hemolysins for sheep erythrocytes. Goebel and Adams (60) have recently isolated a lipopolysaccharide complex from pneumococci which exhibited powerful Forssman antigen activity as demonstrated by hemolysis inhibition tests (see below). These workers (60) also showed that the polysaccharide part of this complex antigen was identical with pneumococcal "C" substance, the somatic polysaccharide present in all types of pneumococci. The extensive literature on Forssman antigens has been reviewed by Buchbinder (61). Extensive chemical and immunological studies on the Forssman antigen were carried out by Brunius (62).

The Forssman antigen has been found to be associated with tissue particles of very high molecular weight. All of the Forssman activity can be sedimented from tissue extracts in the ultracentrifuge in one hour at 27000 r.p.m. (63). The Wassermann antigen is associated with particles of similar size as well (64). Autolysis of the tissues is accompanied by destruction of the Forssman antigen (63).

Titration of hemolysin are usually carried out with a standard dose of erythrocytes (100 million in a volume of 0.1 ml.) and 0.2 ml. of 1:20 fresh guinea pig serum as complement (cf. p. 110). The

reproducibility of the titer can be increased by using a definite dose of complement, i.e., two 100 per cent or four 50 per cent units, instead of an arbitrary volume of guinea pig serum. In this form the test requires a preliminary determination of the complement unit using a fixed amount (e.g. 4 units) of a sample of hemolysin arbitrarily selected as reference standard.

Hemolysis inhibition test: The hemolysis inhibition method introduced by Brahn and Schiff (65), is based on the fact that hemolysis of sheep cells by specific antibody and complement is inhibited, owing to competitive action, by antigens reactive with the antibody. It may be illustrated using the example cited above, namely the Forssman antigen in human erythrocytes of blood group A. Polysaccharide-amino acid complexes have been obtained which possess blood group A activity, as determined by their capacity to inhibit agglutination of group A erythrocytes by sera from group O or B individuals (cf. II-9, IV-53). These substances also display Forssman antigen activity, as may be demonstrated by their capacity to inhibit the lysis of sheep erythrocytes with complement and the Forssman antibody in rabbit antisera to A cells.

Procedure: The technic of the test, modelled after Brahn and Schiff (65) and Brunius (62) involves a preliminary titration of the Forssman antibody with the standard dose of sheep erythrocytes and two 100 per cent or four 50 per cent units of complement (see above). Two-tenths ml. of a series of progressive dilutions of the antigen to be tested for Forssman activity is then mixed with 0.1 ml. of an antibody dilution containing 2 units of hemolytic activity as determined in the preliminary test. After 5 minutes at room temperature there is added to each tube 0.1 ml. of a suspension of washed sheep erythrocytes containing 100 million cells, and after another 5 minutes at room temperature 0.2 ml. of a dilution of fresh guinea pig serum containing 2 units of complement is added. The tubes are then incubated for 30 minutes at 37° C. Results are recorded in terms of degree of lysis as in complement fixation tests. The end-point is taken as the minimum amount of Forssman antigen giving complete inhibition of hemolysis.

Another technic modelled after Landsteiner (66) and Morgan (67) involves a different sequence of operations. Decreasing amounts of the antigen are mixed with rabbit antiserum to human A cells containing 2 units of Forssman antibody and two 100 per cent or

four 50 per cent units of complement. After one hour's incubation at 37° C. the sheep cells are added and the tubes again incubated for ½ hour at 37° C.

In both forms of the test, but especially in the latter procedure, the ability of the Forssman antigen-antibody complex to fix complement must also be considered. One can therefore regard inhibition of hemolysis as due to complement fixation as well as to competition between Forssman antigen and sheep cells for a limited amount of hemolytic antibody.

Each hemolysis inhibition test includes a control tube containing saline instead of A-substance, and this should show complete lysis. As in complement fixation tests, it is necessary to ascertain that the preparation of Forssman antigen used is not anticomplementary. This may be done by incubating the largest dose of antigen to be employed with complement. Sheep cells sensitized with hemolysin are then added, and the tubes again incubated for ½ hour. Under these conditions the antigen does not react with hemolysin, so that unless the antigen is anticomplementary, the complement remains free to lyse the sensitized cells. As an additional control, a tube containing Forssman antigen, hemolysin and sheep cells, but no complement, should be included to verify absence of any lytic properties in the antigen.

As a control on the specificity of the hemolysis inhibition, Brunius (62) uses a guinea pig anti-sheep erythrocyte serum (isophile serum). This serum also hemolyzes sheep cells in the presence of complement but its action is not due to Forssman specificity and can therefore not be inhibited by Forssman antigen.

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CHAPTER 5

ANAPHYLAXIS AND ALLERGY

Anaphylactic shock is one of the most dramatic indications of the combination of antigen with antibody. Although initial quantitative data on the amounts of antigen and antibody required for its production have only recently been obtained (1-3), numerous workers have used anaphylaxis to study the specificity of antigen-antibody reactions (4, 5), to determine small amounts of specific polysaccharides (6) and of proteins, and to disclose differences between antibodies produced in various animal species (7, 8). Anaphylactic shock can be elicited in numerous animal species. The guinea pig, however, responds most rapidly and characteristically, and is the animal of choice for studies on anaphylaxis. Detailed reviews of early literature in the field may be found in references (9-11).

ACTIVE ANAPHYLAXIS

If a guinea pig receives an initial injection of a foreign protein such as crystalline egg albumin, no harmful effects are noted. After an interval of at least ten days to three weeks, an intravenous injection of egg albumin into the same animal will elicit an anaphylactic response, characterized by restlessness, chewing, rubbing of the nose, dyspnea, convulsive movements and convulsions, frequently terminating fatally.

The first injection of antigen is known as the sensitizing injection and it is generally accepted that during the time interval which must elapse, antibodies are formed, some or all of which remain fixed to the tissue cells. These antibodies then react with the antigen upon subsequent injection and initiate the anaphylactic response. The second injection is known as the shocking dose. For uniform results, the shocking injection is best administered intravenously.

Guinea pigs have been actively sensitized to a wide variety of proteins varying in molecular weight from about 40,000 (crystalline egg albumin) to 6,800,000 (hemocyanin) and about 30-40 million (tobacco mosaic virus) (12, 13). Sensitization has been induced with as little as 10^{-6} ml. of horse serum containing about 0.07 micro-

gram protein (14) or with 0.05 microgram crystalline egg albumin (15). For uniform sensitization, however, larger amounts are required. Sensitizing injections may be given intracutaneously, subcutaneously, intraperitoneally or intravenously. A single subcutaneous injection of 0.01 to 1 mg. of protein per guinea pig is generally satisfactory. Better results may be obtained frequently by the use of a series of 3-5 subcutaneous injections at 4-7 day intervals. Sensitization has also been effected via the placenta, the gastro-intestinal tract and by inhalation of antigen (9-11) dispersed as fine droplets (11). Once sensitivity has been induced it may persist for periods of six months or more (9, 10).

Recent studies of Landsteiner and his associates have shown that guinea pigs may be sensitized by a series of intracutaneous injections to simple chemical substances such as picryl chloride, 2:4 dinitrochlorobenzene, etc. (16) so that they will show typical symptoms of anaphylaxis upon subsequent injection of these materials, conjugated to protein. Skin sensitization (see below) also resulted. The sensitizing power of such simple chemical substances is attributed to their ability to combine with protein in the host to form complete antigens.

The shocking dose of antigen, if of suitable size and if administered intravenously, should produce anaphylactic death within 2-10 minutes. The amount of antigen necessary for fatal shock is considerably larger than the minimal amounts which may be used for sensitization and may vary from 0.1 to 10 mg. with different proteins. Anaphylaxis may be induced by intraperitoneal injection of the antigen but this method is less sensitive and less reliable in that much larger amounts of antigen are required, the development of symptoms is delayed and prolonged, and uniform results are more difficult to obtain. Induction of anaphylactic shock in sensitized guinea pigs by inhalation of finely dispersed particles of antigen is readily accomplished (11, 17, 18, 18a) and may in some instances even be as satisfactory for eliciting anaphylaxis as intravenous injection.

Tissues of sensitized animals can be used to demonstrate sensitivity. Thus uterine or intestinal strips from sensitized guinea pigs placed in a bath containing Ringer's solution contract when antigen is added. This contraction may be recorded with a kymograph (Schultz-Dale technic).

After receiving a sub-lethal shocking dose of antigen, actively sensitized guinea pigs become refractory for a period of about several days or weeks and are said to be desensitized (9-11).

Anaphylaxis, as a manifestation of the combination of antigen with antibody *in vivo*, shows the same degree of specificity as do other antigen-antibody reactions, as, for example, the precipitin reaction. Thus Dakin and Dale (4) sensitized female guinea pigs by injection of 1 mg. of either hen or duck ovalbumin. After 3-4 weeks uterine strips were tested by the Schultz-Dale technic for sensitivity to each antigen. In some instances, sensitization was strictly specific, but more frequently sensitivity to both antigens could be detected. They found, however, that the heterologous antigen could never completely desensitize the uterine strip so that it would no longer react with the homologous antigen.

From the standpoint of obtaining quantitative information, methods using active anaphylaxis are of very limited value. It is at present impossible to determine directly the actual amount of antibody produced by a guinea pig in response to sensitization. Since in other species which have been studied, active immunization may result in as much as a million-fold variation in the antibody response of individual animals, it is obviously difficult to consider one actively sensitized guinea pig as equivalent in antibody content to another, merely because they received the same sensitizing injection. Thus the failure of Dakin and Dale to obtain contractions of uterine strips of some animals sensitized with hen ovalbumin when tested with duck ovalbumin and *vice versa*, may be attributed either to a lower total antibody content in these animals or to the absence of formation of cross-reacting antibody regardless of antibody content. For example, only occasional rabbit antisera to type III pneumococci cross-react with type VIII pneumococcal polysaccharide (11, 9).

Demonstration of active anaphylaxis is of value, however, in establishing whether a given substance is antigenic and also in the detection of very small amounts of impurities. Seastone, Loring and Chester (12) were able to show that preparations of tobacco mosaic virus prepared by four differential ultracentrifugations no longer induced anaphylactic sensitization to normal tobacco protein, indicating the absence of normal tobacco protein in these preparations. Similar studies may be used to detect broth proteins

in washed bacterial suspensions or egg-proteins in purified egg-grown virus preparations. The method, while extremely sensitive, provides only qualitative information and it is not possible to estimate what proportion of impurity may be present if positive results are obtained. It has also been used by Rimington and Van den Ende to demonstrate identity of a serum protein fraction with serum albumin (12a).

PASSIVE ANAPHYLAXIS

Injection of the serum of an actively sensitized guinea pig into a normal guinea pig has been found to sensitize the normal animal so that a typical anaphylactic response occurs if antigen is injected several hours later. The same results may be obtained by injecting the sera of certain other animals hyperimmunized to a given antigen. This phenomenon, so-called passive anaphylaxis, has proven readily applicable to quantitative investigations (1-3) and has led to a clearer understanding of the role of antibodies in the production of anaphylaxis (for earlier studies cf. 9-11, 15, 18, 18a, 19).

While the amount of antibody produced by a guinea pig in response to active sensitization cannot be estimated, the minimum amount of antibody nitrogen required to induce passive sensitization can readily be determined using antisera of known antibody content as measured by the quantitative precipitin or agglutinin method (I, Ch. 2, 3), and it may be inferred that similar amounts of antibody are probably present in actively sensitized animals. Although studies in this field are still in the early stages (1, 2, 3), the broad outlines of the quantitative relationships of antigen and antibody in passive anaphylaxis are already becoming clear.

In studies of passive anaphylaxis it is important, for uniformity in results, that both sensitizing and shocking doses be given intravenously, the former to ensure uniform distribution of antibody throughout the animal's tissues (20) and the latter to effect most rapid interaction of antigen and antibody. Although intracardiac injections are frequently used for eliciting shock, they may occasionally lead to symptoms due to hemorrhage into the pericardium and these may be interpreted as anaphylactic reactions. After some practice intravenous injections of guinea pigs may be performed with precision and dispatch. The technic is described in the appendix.

Tables 1 and 2 show the results obtained (1, 2) by injecting known amounts of rabbit and guinea pig antiovalbumin into guinea pigs (250 ± 40 gms.) and injecting a standard amount (1 mg.) of egg albumin 48 hours later. Studies on passive sensitization with known amounts of type III antipneumococcal rabbit antibody followed by shock elicited with 0.1 mg. type III pneumococcal polysaccharide are also included. It is apparent from these data that a sensitizing dose of as little as 0.03 mg. rabbit or guinea pig antioval-

TABLE 1

Passive Sensitization of Guinea Pigs with Varying Amounts of Rabbit Antibody

Antibody N injected	No. of guinea pigs used	Results			
		Deaths	Severe reactions	Slight reactions	No reactions
Rabbit anti-egg albumin. Guinea pigs shocked with 1 mg. egg-albumin intravenously 48 hours after sensitizing injection					
mg. 0.0019	4	0	0	2	2
0.0038	4	0	1	3	
0.0057	5	0	3	2	
0.0064	1	1			
0.0075	4	0	3	1	
0.0113	6	4	2		
0.023; 0.024	5	3	2		
0.034; 0.036	6	6			
0.048	2	2			
0.060	8	8			
0.072	1	1			
Rabbit antibody to type 3 pneumococcus. Guinea pigs shocked with 0.1 mg. type 3 polysaccharide intravenously 48 hours after sensitizing injection					
0.010	4	1	2	1	
0.020	5	4	1		
0.030	4	4			
0.040	4	4			

From (1).

bumin or antipolysaccharide N prepares the animal for uniformly fatal anaphylaxis when 1 mg. (0.16 mg. N) of ovalbumin or 0.1 mg. of pneumococcal polysaccharide is injected intravenously 48 hours later. With sensitizing doses of 0.006 to 0.025 mg. antibody N anaphylactic reactions occur, but are not always fatal. With smaller amounts of antibody N, fatalities do not occur and only slight or negative reactions are noted. Rabbit anti-egg albumin

and guinea pig anti-egg albumin are equally effective on a weight basis in passively sensitizing the guinea pig.

Tables 3 and 4 show the effect of varying the amount of egg albumin used in shocking guinea pigs passively sensitized with known amounts of rabbit or guinea pig anti-egg albumin. It is apparent that the reaction is quite dependent on the amount of antigen used for eliciting shock.

TABLE 2

Passive Sensitization of Guinea Pigs with Varying Amounts of Guinea Pig Antiovalbumin. Guinea Pigs Shocked with 1 mg. Ovalbumin Intravenously 48 Hours After Sensitizing Injection.

Antibody N injected	No. of guinea pigs used	Results				
		Dead	Severe	Moderate	Slight	Negative
mg.						
0.005	6		2	2	2	
0.006	4		3	1		
0.010	7	5	2			
0.012	4	1	2	1		
0.015	7	6	1			
0.018	4	4				
0.020	8	7	1			
0.024	5	3	2			
0.030	8	8				
0.036	4	4				
0.040	10	10				
0.048	3	3				

From (2). Courtesy of Williams and Wilkins Co.

Comparison of the amounts of antigen and antibody required for anaphylaxis (tables 3 and 4) with those found at the point of maximum precipitation in the precipitin reaction (I, 2) indicates that the stoichiometric proportions of the reactants in each type of test are very different. Only 0.003 mg. of egg albumin N would be required to precipitate the 0.03 mg. rabbit antibody N used to ensure uniformly fatal shock. However, 0.16 mg. of egg albumin N or 50 times this quantity was required for fatal anaphylaxis; 0.016 mg. egg albumin N or five times as much was not sufficient. It

TABLE 3

*Effect of Variation of Antigen (Egg-Albumin) on Passive Anaphylaxis
Produced in the Guinea Pig with Homologous Rabbit Antibody*

Antigen Injected	No. of guinea pigs used	Results			
		Deaths	Severe reaction	Slight reaction	No reaction
Guinea pigs sensitized with 0.0113 mg. rabbit antibody N intravenously					
10 mg.	3	2	1		
1	6	4	2		
0.1	5	1	1	3	
0.01	5	0	1	4	
0.001	5	0	0	2	3

Guinea pigs sensitized with 0.060 mg. rabbit antibody N intravenously

1	8	8			
0.1	5	3	2		
0.01	5	0	2	3	
0.001	5	0	0	4	1

From (1). Courtesy of Williams and Wilkins Co.

TABLE 4

*Effect of Variation of Antigen (Egg Albumin) on Passive Anaphylaxis
Produced in the Guinea Pig with Guinea Pig Antibody*

Antigen injected	No. of guinea pigs used	Results			
		Deaths	Severe reaction	Slight reaction	Negative
Guinea pigs sensitized with 0.024 mg. antibody N intravenously					
mg. 1.0	5	3	2		
0.1	4	1	3		
0.01	4		1	1	2

Guinea pigs sensitized with 0.04 mg. antibody N intravenously

1.0	10	10			
0.1	5	5			
0.01	4				4

From (2). Courtesy of Williams and Wilkins Co.

would appear therefore that anaphylactic reactions with minimal sensitizing doses of antibody have their optima at the extreme antigen excess end of the precipitin reaction (the inhibition zone) where soluble compounds are formed and where all or most of the reactive groups on each antibody molecule are combined with antigen (1). It is possible, however, that only a small proportion of the injected antigen actually combines with antibody during the interval between intravenous injection and the fatal reaction.

TABLE 5

Passive Anaphylaxis with Rabbit Antibody to Tobacco Mosaic Virus

Antibody N for sensitization	Tobacco mosaic virus N used for eliciting shock				
mg.	mg.	mg.	mg.	mg.	mg.
	0.16-0.18	0.22-0.24	0.42	1.2	3.6
0.03	moderate (2)	moderate (1)		severe (1)	fatal (2)
0.10	slight (1)	slight (2)* severe (1)		moderate (1) slight (1)	fatal (1)
0.20			severe (1) doubtful (1)	slight (1) doubtful (1)	

Kabat, E. A., Coffin, G. S., and Smith, D. J.: *J. Immunol.* 1947, **56**: 377. Values in parenthesis represent number of animals.

* One animal had received 0.30 mg TMV N

Table 5 shows data obtained using rabbit antisera to tobacco mosaic virus in inducing passive sensitization. Although only few animals have been used it is apparent that the results are strikingly different from those obtained with the egg albumin system in that much larger amounts of antigen are required for eliciting fatal shock, 3.6 mg. virus N for 0.03 mg. antibody N as compared with 0.16 mg. egg albumin N (1 mg. protein). This is probably related to differences in molecular weight of tobacco mosaic virus and egg albumin, since a given quantity of virus would contain many fewer molecules than the same amount of egg albumin. Since each molecule of tobacco mosaic virus contains more groups reactive with antibody than does egg albumin the relationship between molecular weight and amount of antigen required for fatal shock is not a simple one.

Follensby and Hooker (3) adopted the technic of using known amounts of antibody N in attempts to sensitize guinea pigs pas-

sively with equine antihemocyanin and equine antiovalbumin sera. Their results, shown in table 6, indicated that no sensitization could

TABLE 6
Passive Sensitization of Guinea Pigs with Equine Antisera

Antibody N injected	Antigen N injected	Results		
		Severe	Indefinite	None
Antihemocyanin				
Untreated serum				
mg.	mg.			
0.006	1.9			1
0.012	2.1		1	2
0.12	2.5			1
0.5	5.0			2
0.6	3.1		2	
0.72*	1.0		1	
1.2	3.1			2
Digested serum				
0.05	4.7			2
0.07	4.6			2
0.12	3.6			3
0.12	5.4			1
Anti-ovalbumin				
0.014	0.8	1†	3	
0.014	1.6		1	1
0.014	2.0		1	
0.07	1.6		1	1

* Reverse anaphylaxis.
† Very rapid recovery; symptoms possibly caused by slight pericardial hemorrhage.
As a control 6 guinea pigs were given sensitizing doses of 0.12 to 0.48 mg. rabbit-antihemocyanin N; 4 had fatal anaphylaxis, 1 had severe symptoms, 1 had mild symptoms.
From (3). Courtesy of Williams and Wilkins Co.

be obtained with varying amounts of antibody nitrogen. They also found that sensitization could not be induced with digested serum or with specific precipitates prepared from these equine antisera. With rabbit antihemocyanin 0.12 to 0.48 mg. N passively sensitized 6 guinea pigs so that subsequent injection of 10-47 times the optimal amount of antigen required for precipitation of the antibody *in vitro*, resulted in 4 fatal, 1 severe and 1 mild reaction. The advantages of using this quantitative approach to passive anaphylaxis studies are very great, for it becomes possible directly

to compare results obtained in different laboratories. Previously no knowledge whatever of the potency of the sera used by various investigators was obtainable. In addition it becomes possible to evaluate in absolute terms the effects on passive anaphylaxis of variations in molecular size and combining proportions of various antigens, of differences in sensitizing properties of antibodies prepared in several animal species, and to study passive sensitization in cross-reacting systems, etc. Passive anaphylaxis could also be more precisely standardized for use in the determination of small amounts of antigen (6) by using a known amount of antibody for sensitization. Extension of these studies to other *in vivo* immune reactions should be of considerable importance.

Isolated uterine or intestinal strips from passively sensitized guinea pigs contract upon contact with antigen in a Dale bath. Uterine strips from female guinea pigs sensitized by intravenous injection of 0.03 mg. antiovalbumin N contracted when 1 mg. of egg albumin was added to the bath containing 50 ml. of Ringer's solution. Contractions also occurred in some instances when 0.02 and 0.01 mg. of antibody N was used in passive sensitization. Since the antibody injected into the guinea pig (wt. 250 gms.) may be assumed according to Freund and others, to be uniformly distributed throughout the body of the animal (20), the amount present in the contracting uterine horn (wt. 75 mg.) would be of the order of 0.01 microgram nitrogen. Thus the minimal amounts of antibody nitrogen necessary to sensitize small amounts of smooth muscle tissue are less than can be detected by any *in vitro* immunological method.

By injection of large volumes (3 ml.) of serum obtained from guinea pigs actively sensitized by a series of intracutaneous injections to simple substances such as picryl chloride and 2:4 dinitrochlorobenzene, Landsteiner and Chase (16) have been able to demonstrate passive sensitization of uterine muscle. Dale tests were carried out by addition of protein conjugates of picryl chloride and 2:4 dinitrochlorobenzene. In some of the sera which could transfer passive sensitivity small amounts of precipitin were found. Although the actively sensitized guinea pigs were skin-sensitive, this sensitivity could not be passively transferred, unlike anaphylaxis. Skin sensitivity is considered in detail subsequently.

Landsteiner and Van der Scheer (16a) have also been able to produce anaphylactic shock and uterine contractions in animals

sensitized with azoproteins using azo dyes prepared by coupling the azo compound to resorcinol (cf. 54).

Reversed passive anaphylaxis: In some instances it is possible to produce passive anaphylaxis by injecting antiserum 4-24 hours after an intraperitoneal injection of antigen. Opie and Furth (21) demonstrated this phenomenon in rabbits using rabbit antibody to horse serum. They could not produce the phenomenon in guinea pigs, but Kellett (22) did this successfully. No studies using single antigen-antibody systems or known amounts of antibody have yet been carried out. Of interest in this connection is the observation of Forssman (23) who found that injection of sera containing Forssman antibodies (rabbit anti-sheep erythrocyte serum) into guinea pigs would produce death with symptoms similar to those of anaphylaxis. This was attributed to the combination of the antibody with the Forssman antigen present in guinea pig tissues. Doubts have been expressed as to whether this phenomenon is actually reversed anaphylaxis (9), especially since Redfern (24) was unable to elicit contractions of isolated uterine strips by addition of sera containing Forssman antibodies. That this constitutes sufficient proof of an essential difference between this phenomenon and anaphylaxis is open to question, since reverse passive sensitization of isolated uterine strips has not been reported, and since contractions of uterine muscle of guinea pigs sensitized to another antigen, tobacco mosaic virus, could not be obtained (12). Fatal anaphylaxis has, however, been produced (25) in guinea pigs by injection of sera from rabbits immunized with alcoholic extracts of horse kidney mixed with pig serum. These sera showed high titers of sheep hemolysins. It is also probable that the differences in symptoms produced by Forssman antibody and those of typical anaphylaxis may be due to variations in the distribution of the antigen or antibody in each case.

Species differences in passive sensitivity: From the quantitative data in tables 1-6, it is apparent that rabbit antisera to several antigens, egg albumin, hemocyanin, tobacco mosaic virus, etc., passively sensitize guinea pigs, whereas horse antisera to hemocyanin and egg albumin fail to induce passive sensitization. Inability of antisera of certain animal species to sensitize individuals of other species passively has been reported by numerous investigators (7-9). A summary of the results is given in table 7

TABLE 7
Passive Transfer of Sensitivity to Various Species with Homologous and Heterologous Antisera

Animal species of antiserum used in attempts to transfer sensitivity	Antiserum to	Passive transfer to:								
		Human	Guinea pig	Rabbit	Dog	Horse	Mouse	Rat	Chicken	Pigeon
Human	Ragweed	+ ¹								
	Diphtheria toxin		+ ²							
	Horse serum		+ ¹⁰							
Guinea pig	Egg albumin		+ ³							
	Diphtheria toxin		+ ²							
	Horse serum		+ ¹¹							
Rabbit	Egg albumin		+ ⁴							
	Pneumococcal polysaccharide		+ ^{4, 5, 6}				+ ¹⁴			
	Hemocyanin		+ ⁷							
	Tobacco mosaic virus		+ ⁸							
	Diphtheria toxin		+ ²							
	Sheep serum		+ ¹³							
	Horse serum			+ ¹⁵			+ ¹⁸		— ¹³	— ¹³
Horse	Egg albumin		— ⁷			+ ¹⁹				
	Hemocyanin		— ⁷							
	Pneumococcal polysaccharide		— ^{5, 8} + ⁹				— ¹⁴			
	Diphtheria toxin		— ²							
Chicken	Horse serum		— ¹⁶							
Rat	Horse serum		— ¹²					— ¹²		
Cattle	Horse serum		— ¹⁷	— ¹⁷						

* Egg white

+ = successful transfer of sensitivity as established by subsequent positive test with antigen

— = failure to transfer sensitivity as established by subsequent negative tests with antigen

¹ Loveless, M. H. J. Immunol. 1941, 41:15.

² Neill, J. M., Sugg, J. Y., and Richardson, L. V. J. Immunol., 1932, 22: 131.

³ Kabat, E. A., and Boldt, M. H. J. Immunol., 1944, 48: 181.

⁴ Kabat, E. A., and Landow, H. J. Immunol., 1942, 44: 69.

⁵ Mehlman, J., and Seegal, B. C. J. Immunol., 1934, 26: 1.

⁶ Avery, O. T., and Tillett, W. S. J. Exper. Med., 1929, 49: 251.

⁷ Follensby, E. M., and Hooker, S. B. J. Immunol., 1944, 49: 353.

⁸ Kabat, E. A., Coffin, G. S., and Smith, D. J. J. Immunol. 1947, 56: 377.

⁹ Bailey, G. H., Raffel, S., and Dingle, J. H. Am. J. Hyg., 1937, 25: 381.

¹⁰ Bailey, G. H., and Raffel, S. J. Immunol., 1937, 33: 75, Am. J. Hyg., 1938, 27: 1.

¹¹ Longcope, W. T., and Rackemann, F. E. J. Exper. Med. 1918, 27: 341.

¹² Gay, F. P., and Southard, E. E. J. Med. Res., 1908, 18: 407.

¹³ Longcope, W. T. J. Exper. Med., 1922, 36: 627.

¹⁴ Doerr, R. Ergebn. Hyg. Bakt. Immunitätsf. u. exp. Therap., 1921, 5: 71.

¹⁵ Friedberger, E., and Hartoch, O. Z. f. Immunitätsf. Orig., 1909, 3: 518.

¹⁶ Mehlman, J., and Seegal, B. C. J. Immunol., 1934, 27: 81.

¹⁷ Pick, E. P., and Yamanouchi, T. Z. f. Immunitätsf., 1909, 1: 676.

¹⁸ Uhlenhuth, P., and Haendel, Z. f. Immunitätsf., 1910, 4: 761.

¹⁹ Gerlach, F. Z. f. Immunitätsf. 1922, 34: 75.

²⁰ Schiemann, O., and Mayer, H. Z. f. Hyg., 1926, 106: 607.

²¹ Ritzenthaler, M. Arch. Int. de Physiol., 1924-25, 24: 54.

(cf. 9); in many cases the actual amounts of antibody used in sensitization is unknown, although the sera were stated to contain large amounts of precipitin. The successful results of Bailey *et al* (26) ((9) table 7) in passively transferring sensitivity to the guinea pig with horse antipneumococcal antibody are in conflict with the findings of numerous other workers. Extension of quantitative studies for evaluation of species differences in the sensitizing power of various antisera would do much to make our knowledge of such differences more precise.

SYMPTOMS OF ANAPHYLACTIC SHOCK IN VARIOUS ANIMALS

Guinea Pig: The symptoms of anaphylactic shock in the guinea pig have been studied by numerous investigators and have been very well described by Seegal (9): "After a pig has been injected intravenously with the . . . shocking dose of antigen, it shows signs of distress within a minute. The hair on the head and back of the neck begins to ruffle. The animal becomes restless, coughs and retches, rubs its nose, and seems to choke. Respirations, which were at first increased in frequency, become slower and labored and soon the animal is gasping for breath and making tremendous inspiratory efforts. The mucous membranes become cyanotic. The animal defecates and urinates. If the anaphylactic shock is destined to end fatally the animal soon becomes weak and rolls over on its side, gives a few convulsive kicks, gasps, and stops breathing. In very severe shock this symptomatic cycle may all be over within five minutes. In a less sensitive animal the symptoms of restlessness, peripheral irritation, and respiratory distress are followed by considerable weakness and a marked drop in temperature. The pig huddles in a corner with hair ruffled and attention centered on its respirations, which are still accomplished with difficulty. After fifteen to thirty minutes it begins to shiver and improvement followed by recovery gradually sets in. The onset of shivering is usually an indication that the body temperature is rising again. If instead of giving a sufficiently large injection of antigen to precipitate acute shock a minute amount is injected, of the order of 0.000001 cc. of serum, guinea pigs develop an elevation of temperature.

"When the shocking dose of antigen is given intraperitoneally the most marked symptom is generally weakness. The animal lies on its side or drags itself around feebly, while the respiratory difficulties are not so marked although never absent. Death may not occur for thirty minutes or more, and indeed animals may finally recover from very grave symptoms."

The blood pressure rises initially from the normal of 80 mm. to 90-140 mm., then gradually (after about 10 minutes) falls to 10-20 mm.

Asphyxia is the immediate cause of death in the guinea pig, and immediately after death the heart is still beating. The lungs are

markedly inflated, due to constriction of the bronchial musculature. Small hemorrhages are common on the under side of the diaphragm and in the viscera.

The Dog: Portier and Richet (27) studied anaphylaxis extensively using the dog as the test animal. Richet's description (28) follows: "In the mildest forms the only symptoms are pruritus, increase in the number of respirations, lowering of the arterial pressure, increased frequency in the movements of the heart, diarrhoea, and tenesmus . . . If anaphylaxis is profound . . . the first symptom is frequent vomiting, so prominent that in a number of cases it develops at the end of ten seconds . . . This symptom is so characteristic that it may be taken as the criterion. The vomit is frothy and mixed with bile; sometimes it is fecal, and sometimes, in severest cases, mixed with blood . . . There is fluid diarrhoea mixed with blood . . . Ataxia suddenly comes on; . . . (the dog) becomes paralytic; drags the hinder part of its body. The pupils dilate and the eyes are dulled, and, after lamentable cries, the animal passes urine and feces, becomes exhausted and insensible . . . Respiration is quickened and dyspnoeic; the arterial pressure is very low, scarcely 4 to 5 cm. of mercury. The heart hurries its beats, which are so weak that sometimes they can scarcely be counted . . . The general condition is serious enough to believe death imminent, but in reality death in less than two hours is extremely rare in the dog."

Rabbit: Anaphylactic shock may be induced but the response in the rabbit is more difficult to elicit than that in the guinea pig. Usually preliminary coughing, irritation and respiratory symptoms are absent. The animal lies with outstretched legs or may fall on its side, gives a series of convulsive movements, often accompanied by passage of urine and feces and dies. Irregular respiratory movements may continue after the heart has ceased beating. At autopsy the characteristic finding is extreme dilatation of the right side of the heart associated with marked obstruction of the pulmonary circulation. Death is attributed to spasmodic constriction of the branches of the pulmonary artery, with rapid dilatation of the right side of the heart and acute heart failure (cf. 9).

Other species: Other animals have been studied less extensively than the guinea pig, dog and rabbit. They include the horse, pigeon, etc. Various species differ in the ease with which anaphylaxis may be induced. For details see (9).

Pathological changes in anaphylaxis: A summary of essential pathological changes found in anaphylaxis occurring in various species as prepared by Seegal (9) is given in table 8. In general, the changes are the result of contraction of smooth muscle and increased capillary permeability.

TABLE 8
*Pathology of Anaphylaxis in Different Species of Animals**

Animal	Congestion and hemorrhage	Edema	Liver	Lungs	Right-sided heart failure
Guinea pig	hemorrhages of stomach, cecum, lungs, heart, etc (Gay & Southard)	lung; skin (Schultz & Jordan; Ramsdell)	occasional local fatty changes	emphysema due to constriction of bronchioles	questionable
Dog	liver, gall bladder, gastro-intestinal tract, lungs, endocardium and pleura, auriculo-ventricular bundle (Dean et al; Richet)	intestinal mucosa (Manwaring, Beattie & McBride)	congestion central necrosis (Weil)	very occasional emphysema	
Rabbit	liver and gastro-intestinal tract (Scott)	slight of lung	marked engorgement of intralobular capillaries, central veins and portal vein	very occasional emphysema. Proliferation & phagocytosis, endothelial cells (Domack)	marked; due to constriction of pulmonary arterioles (Drinker & Bronfenbrener; Gilbert)
Rat	gastro-intestinal tract, lymph gland, etc. (Parker et al)		congestion	very occasional emphysema	
Mouse	intestine and stomach (Ritz)			moderate emphysema (Schultz & Jordan)	present

* From "Anaphylaxis" by B. C. Seegal, in "Agents of Disease and Host Resistance" by F. P. Gay. Modified as suggested by Dr. Seegal. Courtesy of Charles C Thomas, Publisher, Springfield, Ill. (as given in Boyd, W. C., *Fundamentals of Immunology*, Interscience Press, New York).

LOCAL MANIFESTATIONS OF ANAPHYLAXIS AND ALLERGY

In addition to the typical systemic anaphylactic reactions and the contraction of isolated smooth muscle, such as uterine or intestinal strips, there are several other types of response which may be elicited by the combination of antigen and antibody in localized areas of tissue. These reactions may generally be induced in several ways. Antigen may be injected locally into an actively or passively sensitized animal or a small tissue site may be sensitized and antigen may subsequently be injected either locally or systemically.

Arthus phenomenon: This type of response was first described by Arthus (29) who observed that rabbits injected repeatedly with horse serum, developed localized areas of inflammation and necrosis from the later injections, although no response whatever had

resulted from initial injections. The inflammatory response begins after several hours and reaches a maximum in several days. Although originally considered as a hypersensitivity of the skin, it has since been shown that the same phenomenon of inflammation and necrosis may be induced in the lung, heart, testis, brain, joints, kidney and peritoneum (cf. 9). Opie was able to transfer local skin sensitization of the Arthus type in rabbits by injection of precipitating rabbit anti-horse serum or rabbit antiserum to crystalline egg albumin (30). He found that large amounts of antiserum were required and that in both actively and passively sensitized animals there was a close correlation between the precipitin content of the serum and the occurrence and severity of the inflammation. Opie was able to effect desensitization of the skin to the Arthus phenomenon by use of massive doses of antigen, sufficient to remove all of the circulating precipitin, provided a pure antigen such as crystalline egg albumin was used (30). He also produced a reversed Arthus reaction, by injection of antiserum into the skin of a rabbit one day following an intravenous injection of antigen (30).

Some quantitative data are available on the amounts of antibody and antigen necessary to produce Arthus reactions in the rabbit. Culbertson (30a) found that mild Arthus reactions could be elicited in actively immunized rabbits when the level of circulating antiovalbumin N was 0.08 mg. per ml. or more. With levels of 0.12 to 0.16 mg. antiovalbumin N per ml., a slough was invariably produced. The degree of severity of the reactions correlated closely with the amount of circulating antibody.

Culbertson (30a) also produced Arthus reactions passively with a serum of known antibody content. In an extension of these studies, Fischel and Kabat (30b) found that minimal Arthus reactions could be produced regularly in rabbits by intracutaneous injection of 25 micrograms of antiovalbumin N followed after one-half hour by injection of ovalbumin. The intensity of the reaction was independent of the route of administration of antigen, comparable results being obtained by intravenous administration or by direct intracutaneous injection of antigen into the site containing antibody. Reversed local Arthus reactions of the same intensity were elicited by local or intravenous injection of antigen followed by local injection of antibody. In direct Arthus studies, if the antibody was injected intravenously, one to two mg. antibody N were required

to yield a positive reaction on subsequent injection of egg albumin at a single site.

By a comparison of the quantity of antibody nitrogen required to elicit a local Arthus reaction with that present in a sensitized strip of guinea pig uterine muscle of comparable weight, the passive Arthus reaction in the rabbit appears to require many times more antibody than does a passive local anaphylactic response in the guinea pig.

Arthus reactions vary in the ease with which they may be produced in different animal species, and Opie has related this variation to the ability of the species to produce precipitins. The rabbit is the animal of choice. Successful results have also been obtained in the guinea pig; the rat and the dog appear to be refractory (30). Arthus type reactions have been observed in the human (cf. 9).

Of fundamental importance is the finding of Rich and Gregory (31 cf. 31a) that periarteritis nodosa could be produced in rabbits by the injection of large quantities of horse serum (10 ml. per kilo.). These animals were skin-tested immediately before sacrificing and all showed Arthus reactions and at autopsy showed definite periarteritis nodosa. The authors concluded both from their animal studies and from clinical studies that periarteritis nodosa was a manifestation of anaphylactic hypersensitivity similar to the Arthus phenomenon (31). The significance of these observations lies in their indication of the nature of the more severe manifestations of hypersensitivity.

LOCAL SKIN SENSITIVITY

Intracutaneous injection of small amounts of an antigen such as ragweed pollen extract into a hypersensitive human induces within 10-15 minutes a skin reaction characterized by a wheal and erythema. This technic is widely used clinically to identify the substances to which individuals are sensitive. This type of skin sensitivity was conclusively shown to be associated with an antibody when Prausnitz and Kustner (32) were able to produce passive sensitization of normal human skin by intradermal injection of serum from a sensitive patient and to elicit a typical wheal and erythema by subsequent injection of antigen into the same sites. This development made it possible to measure variations in amounts

of skin-sensitizing antibody by dilution or neutralization tests in normal human skin. Injection of mixtures of antigen and skin sensitizing antibody produce an immediate wheal and erythema. Anaphylaxis, however, is not usually produced when mixtures of antigen and antibody are injected. However, Kulka has reported contractions of uterine strips when antigen-antibody mixtures were added (32b). Reversed local passive sensitization of human skin to horse serum has also been successful using sera from individuals sensitive to horse serum. This reversed reaction was found to be more sensitive than the direct Prausnitz-Kustner reaction (32a).

Recent studies on ragweed sensitivity in humans and in cattle have done much to clarify the antigen-antibody relationships in human hypersensitivity, and to demonstrate that the experimental findings in human allergy could be duplicated in animals. This suggests that there is no essential difference in immunological mechanisms between sensitivity in humans and in animals (33). Cook Barnard, Hebal and Stull (34) and later Harley (35), observed that the sera of ragweed-sensitive patients who had been treated by subcutaneous injections of ragweed extracts showed an increased capacity to neutralize antigen, and, in some instances, were able to inhibit the induction by antigen of a skin response in passively sensitized skin. Parenteral administration of antigen to normal non-sensitive individuals resulted in the formation of antibodies which could not sensitize skin passively but were capable of inhibiting the reaction of passively sensitized skin to antigen (36-37). Skin-sensitizing properties of the sensitizing antibodies were destroyed by heating at 56° C. for 2-5 hours, whereas the neutralizing or inhibiting antibodies were unaffected by heating at this temperature. Little is known about the possible role of complement in the action of skin-sensitizing antibodies. Skin-sensitizing antibody in normal human skin remains at the site of injection for as long as a month, whereas the inhibiting antibody disappears after a few hours (38). Skin-sensitizing antibody alone could be obtained by using serum of ragweed-sensitive patients who had not been treated with antigen. Serum from normal individuals injected with ragweed or heated serum from treated ragweed-sensitive patients provided inhibiting antibody alone. With these reagents, methods for the measurement of each of these two antibodies by

passive transfer technics were developed by Loveless (38), and have been applied in studies on the relation of thermostable antibody and clinical immunity in hay fever (39). These two types of antibody have also been found in the serum of a patient sensitive to insulin (40), and Weil and Reddin (33) have been able to demonstrate both types of antibodies in the sera of cattle. As in human sera, the skin-sensitizing antibody was destroyed after 2 hours at 56° C. and the inhibiting antibody was thermostable (33). Rabbit antisera to ragweed pollen have also been found to inhibit the reaction of antigen with sensitized areas of skin in cattle (33). Rabbit and guinea pig antibodies to ragweed pollen, however, are able to sensitize human skin (41, 42). Several investigators have been able to produce passive transfer reactions in guinea pig skin using precipitating rabbit antisera to horse serum, ragweed, egg albumin (43), and pneumococcal polysaccharide (8, 43). Sheep antibodies to ragweed pollen have been found to inhibit the Prausnitz-Kustner reaction (44).

Both ragweed and cottonseed extracts contain several antigens and sensitivity to at least two of these has been observed in each instance (45, 45a). Purification of the various antigens would do and has done much to facilitate studies on hypersensitivity (cf. 46).

An important gap in our knowledge of hypersensitivity has been the lack of clarity about the conditions responsible for the production of skin-sensitizing antibodies. This gap has been considerably narrowed by the studies of Landsteiner and Chase who were able to induce both skin and anaphylactic sensitivity in guinea pigs by intracutaneous (16) injections of simple chemical substances, such as picryl chloride and 2:4 dinitrochlorobenzene. If administered together with killed tubercle bacilli in paraffin oil (47, 48) skin sensitization by intraperitoneal injections may also be induced to these simple substances or to protein conjugates prepared from these substances. A highly successful technic using an emulsion of antigen and killed tubercle bacilli in "aquaphor" and paraffin oil has been developed by Freund and McDermott (49). With guinea pig antisera to ragweed and to horse serum, prepared by this technic (49), Chase (43) was able to transfer local passive skin sensitivity to normal guinea pigs. Anaphylactic sensitivity could also be induced by passive transfer with these sera (43). Quantitative studies of the amounts of antibody required for transferring skin sensitivity

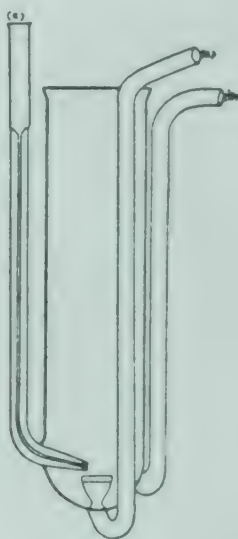
and anaphylactic sensitivity would provide valuable information about the identity of these two types of antibody. Studies on the mechanism of the action of adjuvants, such as tubercle bacilli, would also be of great consequence. This technic has recently been used for the rapid production of acute disseminated encephalomyelitis in *rhesus* monkeys (55, 56). By injection of rabbit or monkey brain or monkey spinal cord as an emulsion with aquaphor, paraffin oil, and killed tubercle bacilli, disseminated encephalomyelitis could be produced with but one to three injections within 3-6 weeks, in contrast to the earlier findings that 30 to 100 injections of aqueous or alcoholic extracts of rabbit brain and intervals of from 3 to 13 months were required before onset of symptoms (57, 58).

Delayed skin sensitivity reaction: This type of skin sensitivity is associated with bacterial infections and notably with tuberculosis. For example, injection of tuberculin in normal guinea pigs produces no reactions. However, a marked inflammatory response results which may become generalized and prove fatal if it is given to tuberculous guinea pigs (9-11). This reaction to tuberculin differs from the usual wheal and erythema type in that depending upon dosage, symptoms do not appear for several hours and reach maximum intensity after 24 hours. When fatal reactions occur, the symptoms do not resemble anaphylactic shock. This type of sensitivity may also be induced by killed tubercle bacilli (50, 51). Studies on the physical properties, antigenicity and intracutaneous activity of tuberculins may be found in references (49a, b). Tuberculin type of sensitivity has been produced with egg white and horse serum by injection into the lesions of tuberculous animals (52). However, passive transfer of the tuberculin type of sensitivity with serum has never been successful (9-11). Landsteiner and Chase (53) succeeded in transferring this delayed type of reaction by intraperitoneal injection of washed cell exudates from guinea pigs which had been immunized by injection of picryl chloride conjugated with guinea pig stromata together with tubercle bacilli (48), or by using the adjuvant mixture of Freund and McDermott (49). The effect was abolished if the cells were destroyed by moderate heating; large amounts of cell exudate were required. Without further studies the relationships of the tuberculin reaction to other types of skin sensitivity and to antibodies can not be clearly defined.

PROCEDURES

Active and passive anaphylaxis: The previous discussion in this chapter should provide adequate information about the amounts of antigen or antibody used for sensitization, of antigen for eliciting shock and of the routes of injection. The technic of intravenous injection of guinea pigs is given in the appendix.

In attempting to sensitize actively to an antigen which has not previously been studied, multiple injections should be used, if success does not follow a single injection. With passive sensitization, more precise data may be obtained by using known amounts of antibody.



Courtesy of Williams and Wilkins Company.

FIG. 8. Tissue-Bath used for isolated muscle-strip tests. (a) Air inlet with sintered glass disc. (b) Aspirator-connection, for emptying bath. (c) Addition tube for antigen-solution. *From (54)*

Schultz Dale technic: For carrying out experiments on isolated intestinal or uterine muscle, a tissue-bath such as that described by Campbell and McCasland (54) and shown in fig. 8 is suitable. It consists of a cylindrical glass tube 150 mm. long and of 25 mm. inside diameter, open at its upper end and sealed and rounded at the bottom. Into the lower end are sealed two 6 mm. glass tubes, whose external portions are bent and extended close alongside the outside wall of the cell to its top. One tube (a) is connected within the cell to a 1 cm. sintered glass disc, and serves to bubble air into the solution. A second tube (b) is connected to suction and serves to

remove the solution. The third tube (c) is a capillary of 1 mm. inside diameter for introducing solutions to be tested into the cell. Its opening inside the cell is just above the sintered glass disc. A piece of glass tubing 3 cm. long and 7 mm. inside diameter is sealed to the upper end of the capillary to serve as a funnel (fig. 8). The muscle strip is supported by a straight glass rod with two curved prongs at one end.

Sensitized guinea pigs are killed by a blow on the head and the small intestine or uterine horns removed. Uterine strips may be removed surgically without sacrificing the animal. Tissues should be kept moist. The intestine is cut into strips of suitable size and the contents removed by washing with salt solution at 37° C. Longitudinal intestinal strips about 3 cm. long or a single uterine horn are tied with thin thread at each end. A loop is made at one end to attach to the glass rod which supports the muscle strip, and the other end is attached to the lever of a kymograph. Two muscle strips may be used simultaneously by connecting each to a separate lever, one advantageously obtained from a normal animal as a control.

The tissue bath is equilibrated in a water bath at 37° C. and is filled with a known volume of Ringer Locke balanced salt solution prepared as follows: 0.9 per cent NaCl, 0.024% CaCl_2 , 0.042% KCl and 0.01-0.03% NaHCO_3 . Some workers use only one-half the calcium chloride concentration (54).

Air is bubbled through the solution, the muscle strip is introduced and connected to the kymograph lever. After equilibration, and if no spontaneous contractions occur the shocking dose of antigen is introduced and the contraction recorded on the kymograph drum. The solution may then be removed by suction and the bath washed with the salt solution, refilled and another test made, with antigen, histamine, etc., as desired.

A single intestine furnishes material for many separate tests. Intestines immersed in salt solution may be kept in the refrigerator for 12-24 hours before use (54).

Uterine strips from animals in oestrus should not be used, and any uterine strips which show spontaneous contractions should be discarded.

Assay of skin sensitizing and thermostable antibodies by

passive transfer studies in humans (cf. 38): Sterile technic is used throughout.

Reagents: All sera should be sterile and Wassermann negative.

A. Serum from untreated hay fever patients.

B. Sera from normal individuals immunized with ragweed pollen, or from ragweed sensitive individuals after injection of pollen extracts.

C. Extracts of defatted pollen preserved with 0.4 per cent phenol and standardized on the basis of protein nitrogen content. 1 unit is defined as 0.01 microgram protein N.

A neutralization technic is used. The serum to be tested is mixed in equal volumes with progressive dilutions of antigen and 0.1 ml. of each mixture is injected into the skin of a normal human subject. As a control one site is injected with a mixture of serum and saline instead of antigen and a second site with saline. Comparisons are usually made by a series of injections on the back of the test subject. The resulting wheal reactions are recorded as soon as any visible non-specific reaction due to the serum has subsided—usually 15 to 45 minutes after the mixtures are injected. After a selected interval, which may vary from several hours to days (6 or 24 hours is usually most convenient), each site is tested by intradermal injection with a 1 ml. tuberculin syringe or preferably with a quarter ml. syringe of 0.025 ml. of a dilution of antigen containing 1000 units per ml. Reactions appear within 15 minutes at the sites prepared with mixtures containing insufficient antigen to neutralize the skin-sensitizing antibody completely. The titer of the serum is expressed in units of the antigen originally present in the site which on testing as above with the antigen solution just gives a doubtful or weakly positive response.

A typical series of results using a serum from the same ragweed sensitive patient before and after treatment is given in table 9 (38). It is apparent that after treatment, reactions to the initial mixtures injected were very slight unless much larger amounts of antigen were used, as compared with the amounts required for the serum obtained before treatment. This difference is attributable to neutralization of the antigen by thermostable antibody (38). Maximal reactions to the initial mixtures occurred with 1500 units of antigen using the serum after treatment whereas only 200 units were required with serum before treatment. Similarly when the presence of

TABLE 9

Neutralization of Skin-Sensitizing Antibody in Serum of a Ragweed-Sensitive Patient Before and After Treatment as Tested in Normal Human Skin

Strength of antigen in units per ml.	Unheated Serum				Serum A + heated Serum B	
	Before treatment (A)		After treatment (B)		Initial Response	Intracutaneous test after 6 hours
	Initial Response	Intracutaneous test after 6 hours	Initial Response	Intracutaneous test after 6 hours		
25	++	+++	=	++++		
50	++	++±			0	+++
100	++	++	=	+++±	0	+++
200	++++	+			0	+++±
300	++++	=				
500			+	++±		
1000			+±	++	+±	+±
1500			+++	+±	+	=
2000			+++	0	+±	0
Saline	=	++++	0	++++	+	+++

From (38). Courtesy of Williams and Wilkins Co.

residual antibody was tested for, end points at between 1500-2000 units and 300 units respectively were obtained in good agreement with those obtained by the first reading.

Assay of the amounts of thermostable antibody in sera from immunized normal individuals or from treated ragweed patients is carried out using a serum from an untreated hay fever patient which has been standardized by determining its combining capacity for antigen as above. The skin-sensitizing antibody in the sera of treated patients is destroyed by heating at 56° C. for four hours. Complete destruction of skin-sensitizing antibody should be checked.

A series of mixtures is then prepared containing one volume of antigen in varying concentrations, one volume of standardized pre-treatment serum and one volume of the serum to be assayed for thermostable antibody, and 0.1 ml. of each mixture is injected into normal human skin. Control sites of each serum and one of saline are injected as above. The initial wheals at each site are read after

15 to 45 minutes. Six (or 24) hours later 0.025 ml. of a solution containing 1000 units of antigen per ml. is injected into each site and the resulting wheals again read after 15 minutes. The last two columns in table 9 show that the mixtures of pre-treatment serum, heated post-treatment serum and antigen behave similarly to unheated post-treatment serum and antigen. Negative or slight initial reactions with the smaller amounts of antigen are obtained indicating that the antigen was prevented from combination with the skin-sensitizing antibody. When tested six hours later a \pm reaction was noted at the site which had received the mixture containing 1500 units of antigen indicating almost complete desensitization in agreement within experimental error with the value obtained for the unheated post-treatment serum.

Assay of skin sensitizing antibodies may also be carried out by measuring the highest dilution capable of passively sensitizing human skin, but this technic is not suitable for assay of the thermostable antibody.

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CHAPTER 6

ANTIBODIES AND THEIR CHARACTERIZATION

The development of chemical methods for the preparation of antibodies in a state approaching purity has ended the controversy about their protein nature. Detailed procedures for the purification of antibodies are given in Sec. IV, 43. Consideration of the physicochemical and immunological properties of these purified antibody preparations, in comparison with their behavior in the original serum before purification, is of importance to the immunochemist in that it provides information about possible alterations in the antibodies as a result of purification.

PROPERTIES OF PURIFIED ANTIBODIES

Physico-chemical properties: Table I summarizes available physico-chemical data on purified antibody preparations obtained from antisera of various animal species by methods described in Sec. IV, 43. The molecular weights and frictional ratios given were calculated from sedimentation and diffusion measurements (cf. III, 26, 27) and the iso-electric points were obtained from electrophoretic measurements (cf. III, 25). The purified antibodies are generally homogeneous in the ultracentrifuge and fall into two groups with respect to molecular weight. Horse, cow and pig antibodies to the type-specific pneumococcal carbohydrates, rabbit hemolysins for sheep erythrocytes, human isoagglutinins and a portion of human Wassermann antibody all have a molecular weight close to 1,000,000, whereas rabbit, monkey and human antipneumococcal antibodies, horse diphtheric antitoxin, and a large proportion of human Wassermann antibody have molecular weights identical with those of the gamma globulins of their respective species. In addition, purified antibodies obtained from many horse antipneumococcal sera have been found to contain appreciable amounts of inhomogeneous materials of lower sedimentation constants, which possessed antibody activity. These will be discussed in detail below. A small proportion of monkey antipneumococcal antibody may also be of high molecular weight since ultracentrifugal examination of 15 per cent NaCl extracts of

TABLE 1
Physical Constants of Purified Antibody Solutions Prepared From Animal Antisera

Animal Immunized	Purified Antibody	% protein in solution examined in the ultracentrifuge	Sedimentation Constant S_n	Diffusion Constant $D_{20} \times 10^7$	Molecular Weight	Frictional Ratio f/f_0	Isoelectric point
			Svedbergs	cm ² /sec.			pH
Horse	Pneumococcus (1,2,3)	0.22	19.3	1.80	920,000	2.0	4.4
	Diphtheria antitoxin (4)		7.2	3.90	184,000	1.4	
	Antitoxin digested with pepsin (5)		5.7	5.0	113,000	1.3	
	Antitoxin digested with trypsin and crystallized (6, 7)		5.5	5.7	90,000	1.23	7.0
Cow	Pneumococcus (2,3)	0.64	18.1	1.69	910,000	2.0	4.8
Pig	Pneumococcus (2,3)	0.58	18.0	1.64	930,000	2.0	5.1
Rabbit	Pneumococcus (1,2,3)	0.19	7.0	4.23	157,000	1.4	5.8
	Egg albumin (1,2,3)	0.53	6.5	3.75	165,000	1.6	5.8
	Sheep red cells (8)		18.9*				
Monkey	Pneumococcus (2,3)	0.31	6.7	4.08	157,000	1.5	
Man	Pneumococcus (2)	0.39	7.4	3.60	195,000	1.5	
	Isoagglutinin (9a)		19.8*				
	Syphilitic Wassermann (9)	0.2	18.5-19.1† 6.5-7.0				

* From activity measurements on whole serum.

† Deutsch (10) reported a value of 36.5 from activity measurements on whole serum.

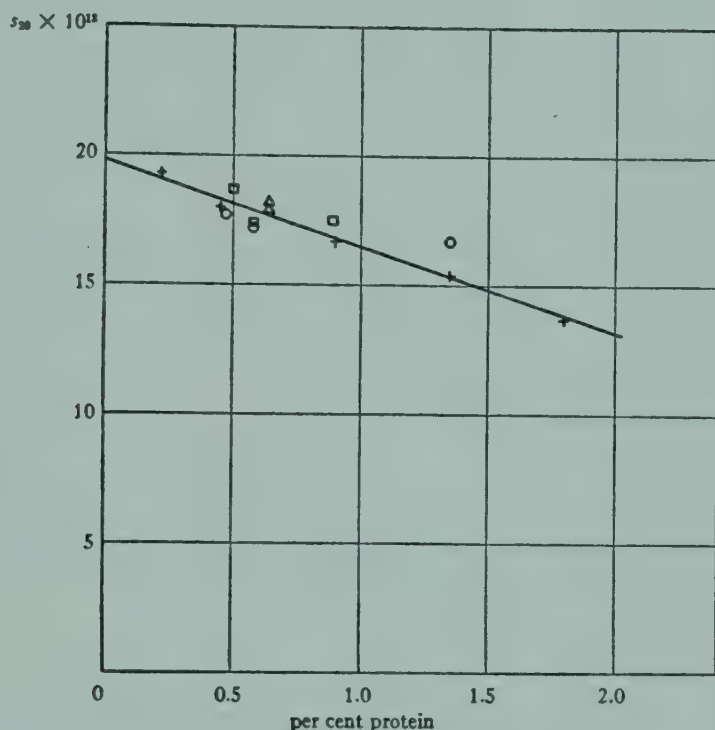
Adapted from (11). Courtesy of Williams and Wilkins Co.

specific precipitates showed small amounts of a component of $S = 17$, which was no longer present after removal of the precipitate formed during dialysis against 0.9 per cent saline (2).

With both kinds of antibodies the sedimentation constants have been found to be dependent on protein concentration over a wide range (2) and the best value for calculating the molecular weight is obtained by extrapolation of the sedimentation constant to zero protein concentration or by correcting for the viscosity of the solution (Lauffer) (see III, 26).

Figure 9 and table 2 show the variation of sedimentation constant with concentration for horse, cow and pig antipneumococcal antibodies. It is apparent that these three species of antibody show the same behavior. The sedimentation constant extrapolated to zero protein concentration is 19.8 and leads to a molecular weight of 990,000 for these antibodies (2). Diffusion constants did not appear to vary with concentration. The molecular weights in table 1 were calculated for the sedimentation constant found at the concentration specified. These large antibody molecules showed marked

double refraction of flow even in 0.1 per cent solution, and had a frictional ratio f/f_0 of 2.0. They are probably rod-shaped particles about $100m\mu$ in length with a ratio of length to width of about 25 to 1 (2).



+, horse 902 E. ○, horse 902 A and B. □, pig. Δ, cow.

FIG. 9. Variation of sedimentation constant of horse, cow and pig antipneumococcal antibodies with concentration. From (2).

TABLE 2

Variation of Sedimentation and Diffusion Constant with Concentration of Solution for Horse Type-Specific Antipneumococcal Antibody

Concentration, % protein	0.22	0.45	0.90	1.35	1.80
S_{20} , Svedbergs	19.3	18.0	16.7	15.4	13.7
$D_{20} \times 10^7$, $cm^2/sec.$	1.80	1.83	1.84	1.63	1.62

Data from (2)

Horse type-specific antipneumococcal antibody was found to be stable over a wide range of pH, no appreciable change in activity occurring after 72 hours at pH 1.44 or after 48 hours at pH 10.9. The shape of the sedimentation diagrams was unchanged between pH 3.4 and 10.9 although the value of the sedimentation constant

TABLE 3

Effect of pH on Sedimentation Constant and Antibody Activity of Horse Type I Antipneumococcal Antibody

pH of solution	Concentration of Solutions in Centrifuge: 0.9 per cent protein					
	Total buffer in solution 0.15 M NaCl plus	Time of standing	Number of components in centrifuge	S ₂₀ of main component	Antibody in solution	
				Svedbergs	%	
1.44	NaCl 0.015 M	10 min.	3	19.5	52*	Also small amounts of components S=6, 27 Small amounts of components S=6, 30
	HCl 0.05 M	72 hrs.	3	20.2		
3.41	NaAc 0.01 M	10 min.	1	18.2		Single symmetrical peak Single symmetrical peak
	HAc 0.25 M	48 hrs.	1	17.6		
4.88	NaAc 0.025 M HAc 0.0125 M	10 min.	1	16.5		Single symmetrical peak
7.65	No buffer	10 min.	1	16.7	49	Single symmetrical peak
9.06	Na ₂ B ₄ O ₇ 0.01 M	10 min.	1	16.3		Single symmetrical peak
10.9	Na ₂ HPO ₄ 0.025 M	10 min.	1	14.7	47†	Single symmetrical peak Single symmetrical peak
	Na ₂ PO ₄ 0.008 M	48 hrs.	1	15.3		
12.4	Na ₂ HPO ₄ 0.05 M	10 min.	2	6.7;6.0		Two definite components; complete break down. Also some of a slightly lower component.
	NaOH 0.10 M	92 hrs.	2	4.5		

* Antibody activity after 48 hours at pH 1.44. Neutralized solution showed main component S=18.4 at 0.8 per cent concentration. Some slight breakdown of the molecule was still evident.
† Antibody activity after 72 hours in ice box, pH 10.9.
From (2).

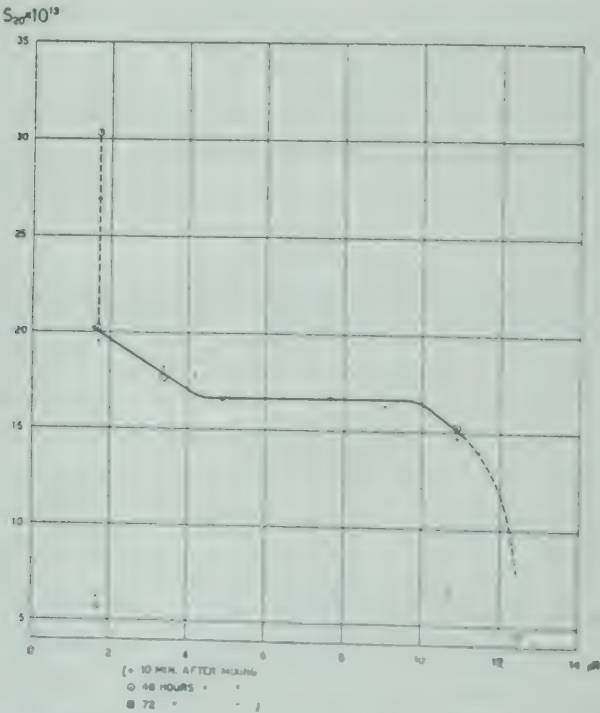


FIG. 10. Effect of pH on the sedimentation constant of horse antibody to the Type I pneumococcal polysaccharide. From (2).

was slightly higher below pH 4.88 and somewhat lower above pH 9.06. Slight breakdown of the molecule without loss of activity was observed at pH 1.44 and complete loss of activity and destruction occurred at pH 12.4. Table 3 and figure 10 summarize data on the pH stability of purified horse antipneumococcal antibody (2).

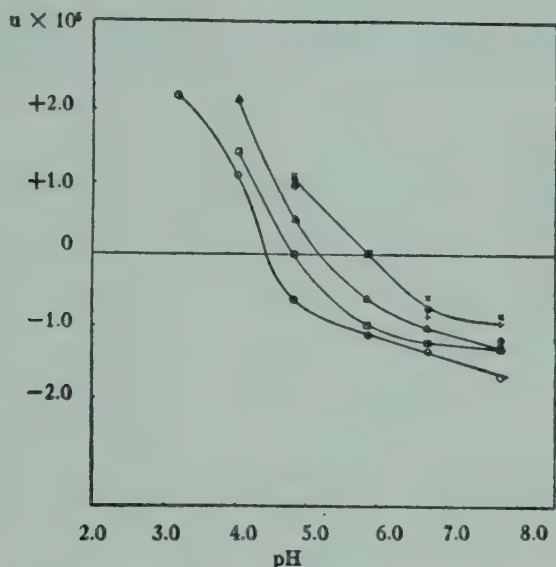


FIG. 11. Mobility of purified antibodies at different pH.

○, horse. □, cow. Δ, pig.
Rabbit 456₂A +; 456₂B x; 431-5 ○.

From (3).

Horse, cow and pig antipneumococcal antibodies all had different isoelectric points and pH mobility curves indicating differences in the net charge of their molecules. The mobility-pH curves of purified antibodies are given in table 4 and figure 11 (3).

Although dissociation with 15 per cent salt has yielded purified type-specific horse antipneumococcal antibody solutions which showed a single homogeneous component of sedimentation constant 17-19, and which were also homogeneous in the Tiselius electrophoresis apparatus with a mobility intermediate between the beta and gamma globulins, in many instances the same method of purification resulted in products showing several components both in the ultracentrifuge and in electrophoresis (2, 3). In the ultracentrifuge these additional components are not homogeneous, vary widely in sedimentation constant and may comprise as much as 50-60 per cent of the total protein in the solution. They possess antibody activity as indicated by tests after removal of the heavy antibody

TABLE 4

Electrophoretic Mobilities of Purified Antibodies at Various pH $\times 10^5$ cm.² sec.⁻¹ volt⁻¹

Antipneumococcus						Anti-egg albumin
pH	Horse, salt dissociated	Cow, salt dissociated	Pig, salt dissociated	Rabbit		Isolated by electrophoresis
				456 ₂ A, salt dissociated	456 ₂ B, barium dissociated	
3.18	+2.21					
4.02	+1.12	+1.43	+2.15			
4.81	-0.63	0.00	+0.49	+1.04	+1.10	+0.95
5.86	-1.12	-0.98	-0.62	0.00	0.00	0.00
6.70	-1.34	-1.20	-1.02	-0.86	-0.60	-0.75
7.72	-1.70	-1.31	-1.30	-0.95	-0.85	-1.20
Isoelectric point pH	4.4	4.8	5.1	5.85	5.85	5.85

From (3).

component in the separation cell and because solutions containing 96 per cent antibody were obtained which showed 60 per cent of low molecular weight material (2). Electrophoretic examination of such solutions showed a substance with a mobility corresponding to gamma globulin in addition to the component of mobility intermediate between the beta and gamma globulins. In some instances antibody of sedimentation constant 18 also had the mobility of gamma globulin (12). The proportions of ultracentrifugally inhomogeneous material and of the gamma globulin constituent were in close agreement (table 5) (2, 3, 11).

The formation of these ultracentrifugally inhomogeneous components was found to be associated with the length of immunization of the animals. Thus, in two instances, a first serum sample yielded ultracentrifugally and electrophoretically homogeneous antibody on purification, whereas the purified antibody from a second sample after one or two additional years of immunization contained ultracentrifugally inhomogeneous materials and a component with the mobility of gamma globulin (table 5).

The second group of antibodies are those with the same molecular

weight as the respective normal gamma globulins. Of these, the horse antitoxins and human Wassermann antibody have a mobility intermediate between the beta and gamma globulins, and the rabbit and monkey antibodies have been found to show the mobility and isoelectric point of normal gamma globulin (table 4) (fig. 11).

TABLE 5

Effect of Prolonged Immunization on Composition of Horse-Antibody Solutions

Horse Used	ULTRACENTRIFUGE		ELECTROPHORESIS	
	S ₂₀	Relative Concentration	$u \times 10^5$	Relative Concentration
	Svedbergs	% of total N	cm ² /volt-sec.	% of total N
902 1st bleeding	17.2	100	-1.7	100
902 2nd bleeding (1 year later)	18.0 12) 10)* 4)	26	-1.7 -0.8	22
Sn 1st bleeding	18.2	100		
Sn 2nd bleeding (2 years later)	18.6 17) 12)* 7)	22	-1.8 -0.9 ₆	
9093 under immunization for several years	17.6 10*	33	-1.6 -0.3	46

* Inhomogeneous.

From (11). Courtesy of Williams and Wilkins Co.

Rabbit antipneumococcal antibodies were more resistant to alkali than horse antibodies, since products obtained by dissociation with barium hydroxide were homogeneous in the former instance, whereas considerable breakdown into materials of low molecular weight occurred with horse antibodies when this method was employed (IV, 43).

The sedimentation constants of purified rabbit, monkey and human antibodies have also been found to vary with the concentration of the solution examined (fig. 12) (2).

By digestion of horse antitoxins with pepsin (13, 14, 5) and trypsin (6, 7) an inert portion of the antibody molecule may be removed leaving antitoxin of higher flocculating activity per unit weight. The molecular weights of these digested antitoxins have been found to be 98,000 and 113,000 (table 1) suggesting that about one-

third to one-half of the molecule is split off by the enzyme. This has been taken to indicate that the antitoxic groupings are distributed asymmetrically about the molecule (4). Trypsin treated diphtheria antitoxin has been crystallized by Northrop (6) after fractionating with ammonium sulfate. Papain has been found to split diphtheria antitoxin into two fragments, one of which flocculates with toxin (14, 15).

Bourdillon (15a) found recently that solutions of antitoxic pseudoglobulin spontaneously split off inert protein on storage for a number

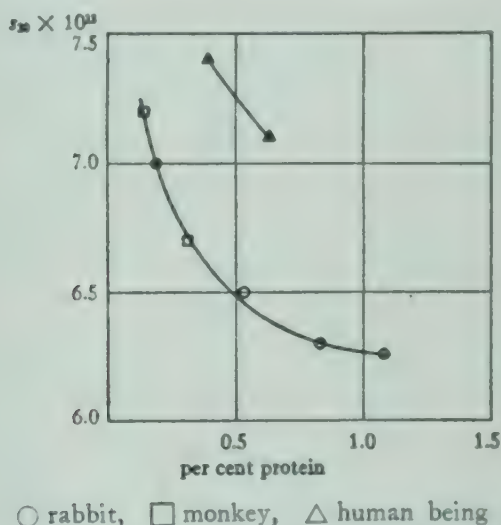


FIG. 12. Variation of sedimentation constant of rabbit, monkey, and human antipneumococcal antibodies with concentration. From (2).

of years in the absence of added enzyme, as evidenced by more rapid flocculation with toxin and by the smaller amount of antitoxin which combined with toxin at the flocculation optimum.

Antigenic properties: The immunological relationships among various antibodies and normal serum globulins have been established by immunizing animals of another species with purified antibodies or with washed specific precipitates, and using their anti-sera to determine by quantitative precipitin or agglutinin methods the amount of antibody removed by various antibody and normal globulin preparations. Thus, Treffers and Heidelberger¹ (16) immunized chickens with washed specific precipitates from Type II antipneumococcal rabbit sera and found that specific precipitates from anti-egg albumin, anti-C, and Type II antipneumococcal rabbit sera all removed the same amount of antibody from the chicken antisera, indicating immunological identity, in addition to

the previously established physicochemical identity, of these rabbit antibodies. The chicken antiserum to rabbit antibodies was species specific and did not react with equine antipneumococcal specific precipitates.

Similar studies have been carried out on the antigenic properties of horse antibodies. Braun (17) was able to sensitize guinea pigs to horse serum by injecting bacteria agglutinated with horse antiserum and Landsteiner and Prasek (18) showed that precipitins for horse serum would remove the agglutinins from antityphoid horse sera. Ando and his co-workers (19) were able to demonstrate by the usual serological technics that pneumococcal anticarbohydrate, typhoid anticarbohydrate and plague antibodies from immune horse sera were almost identical in antigenic properties, but were distinctly different from the antitoxins. With a rabbit serum prepared by immunization with Type II pneumococcal specific precipitates from horse antisera, Marrack and Duff found that normal horse globulin precipitated much less antibody than did a purified antibody solution.

Quantitative studies on the antigenic relationships between horse antibodies have been carried out by Treffers and Heidelberger (20). Quantitative antibody determinations using a rabbit antiserum to washed Type II pneumococcal specific precipitate from horse antiserum showed that the antipolysaccharide in Types I and VIII antipneumococcal and in anti-influenzal horse sera and the anti-C carbohydrate could remove the same amount of antibody nitrogen from the rabbit antiserum. Purified solutions of Types I and II pneumococcal anticarbohydrate also removed the same amount of antibody nitrogen as did the specific precipitates (table 6). Specific precipitates containing horse anti-egg albumin and diphtheria antitoxin, however, removed but a fraction of the antibody from the rabbit antiserum indicating differences in the antigenic properties of these two antibodies (table 6). It is of interest that in addition to differences in reactivity with their respective antigens, horse antibodies of the antitoxic type are antigenically different from those of the precipitin type.

The antigenic relationships between normal horse globulin and antibodies were studied quantitatively using an antiserum to Type II pneumococcal specific precipitates by Treffers, Moore and Heidelberger (12). Electrophoretically separated gamma globulins from

TABLE 6

Maximum Amount of Antibody N Removed after 48 Hours at 0° from 1.0 ml. Rabbit Antiserum to Horse Type II Specific Precipitate by Various Antigens

Test antigen (horse)	Maximum antibody N precipitated per ml. serum
	<i>mg.</i>
Anti-Pn II, high ratio sp. ppt.....	0.52
Anti-Pn II, low ratio sp. ppt.....	0.48
Anti-Pn I, high ratio sp. ppt.....	0.50
Anti-Pn C, high ratio sp. ppt.....	0.50
Anti-Pn C, low ratio sp. ppt.....	0.51
Anti H. influenzae, type B, sp. ppt.....	0.53
Ea-anti-Ea sp. ppt.....	0.26
Diphtheria toxoid-antitoxin floccules.....	0.31
Pn II anticarbohydrate solution.....	0.54
Pn I anticarbohydrate solution.....	0.51
Sp. ppt. from partially digested (pepsin) Pn I anticarbohydrate.....	0.50
Anti-Pn II (rabbit) sp. ppt.....	0
Anti-Pn I (pig) sp. ppt.....	0

From (20)

normal and immune sera showed different antigenic properties. Two preparations of the high molecular weight globulin from normal horse sera behaved differently; one reacted almost to the same extent with the antiserum as did purified antibody solutions, but the other removed only part of the rabbit antibody. Treffers, Moore and Heidelberger (12) have suggested that the antibody molecule of high molecular weight may be a polymer of six normal gamma globulin molecules and that this might account for the cross reaction between the normal gamma globulin with rabbit antisera to specific precipitates.

Digestion of antitoxins with pepsin or takadiastase produces a marked impairment in antigenicity (21, 22) and any slight remaining antigenicity has been attributed to a small amount of residual undigested protein. The precipitin reaction of digested antitoxins with antisera to native antitoxins has not yet been studied.

PROPERTIES OF ANTIBODIES IN HYPERIMMUNE SERA

Physico-chemical properties: In any consideration of the physicochemical properties of antibodies in whole serum, it must always be recognized that changes in the serum proteins other than the production of antibodies frequently result from hyperimmunization. Thus Boyd and Bernard (23) have shown that increases in non-specific globulin occur in addition to the increase due to the antibody itself. The proportions of antibody and of globulin formed vary from one animal to another, so that it is never permissible without direct measurements of the amount of antibody produced or of the given property of the serum before and after removal of antibodies, to ascribe any change in the proteins of hyperimmune serum to the antibody alone. Limitations of electrophoretic and ultracentrifugal analysis are discussed in III, 25, 26 for antisera

TABLE 7
Distribution of Antibody in Rabbit and Monkey Antisera by Electrophoresis

Antiserum used	No.	Antibody by precipitin analysis		Total area under curve	Area γ component unabsorbed	Area γ component absorbed	Antibody area by difference	γ component in unabsorbed serum	Antibody in γ component	Antibody in original serum
		%		cm ²	cm ²	cm ²	cm ²	%	%	%
Rabbit anti-egg albumin	431-5	36.4	Positive side	229.4	119.8	34.4	85.4	52.2	71.2*	37.2
			Negative side	252.4	125.1	29.5	95.6	49.6	76.4*	37.9
Rabbit anti-egg albumin	446	12.3	Positive side	94.9	31.1	18.6	12.5	32.8	40.2	13.2
			Negative side	111.5	32.1	16.7	16.1	28.8	48.0	13.8
Rabbit anti-pneumococcus Type I	517	18.6	Negative side†‡	143.4	80.5	28.2	52.3	56.0	35.0	19.6
Monkey anti-pneumococcus Type III	8-58	6.6	Positive side	206.1	53.7	31.7	22.0	26.1	41.0	10.7
			Negative side	217.6	46.4	28.7	17.7	21.3	38.2	8.1

* Isolation of the component from this serum by electrophoresis yielded a solution containing 76 per cent antibody by analysis.

† Positive side results not used because of unsatisfactory reference scale; base line could not be accurately drawn.

‡ A different dilution was used for absorbed and unabsorbed sera; values given are corrected to the same dilution from (3).

in which only a small proportion of the total protein is antibody.

! The electrophoretic patterns of all hyperimmune rabbit sera which contained amounts of antibody sufficient for precise measurements, showed the antibody to be associated with the gamma globulin. The amount of antibody measured by the decrease in area of the gamma component after absorption of the serum cor-

responded quantitatively within experimental error with the amount determined by the quantitative precipitin method (3). Data of this type with rabbit antisera and with a monkey antipneumococcal antiserum are given in table 7 (3), and the electrophoretic patterns before and after absorption are given in fig. 87 of IV, 43. In the ultracentrifuge, Paic (8) reported that rabbit hemolysins for sheep erythrocytes have a sedimentation constant of 18.9 which would indicate a molecular weight of about 1,000,000. In all other instances, no evidence of an antibody component of molecular weight higher than that of normal gamma globulin has been found in rabbit antisera.

Human Wassermann antibody has been found to have a mobility in whole serum between the beta and gamma globulins by studies

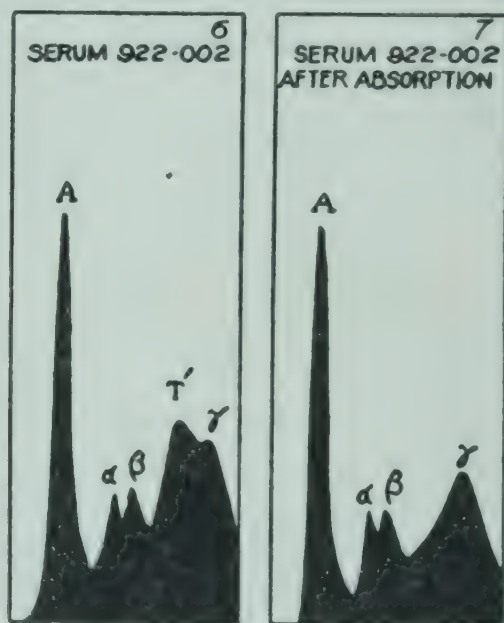


FIG. 13. Electrophoretic patterns of horse antipneumococcal type V serum. (A). Whole antipneumococcal serum. (B). Serum after absorption with type V pneumococcal carbohydrate. *From (26).*

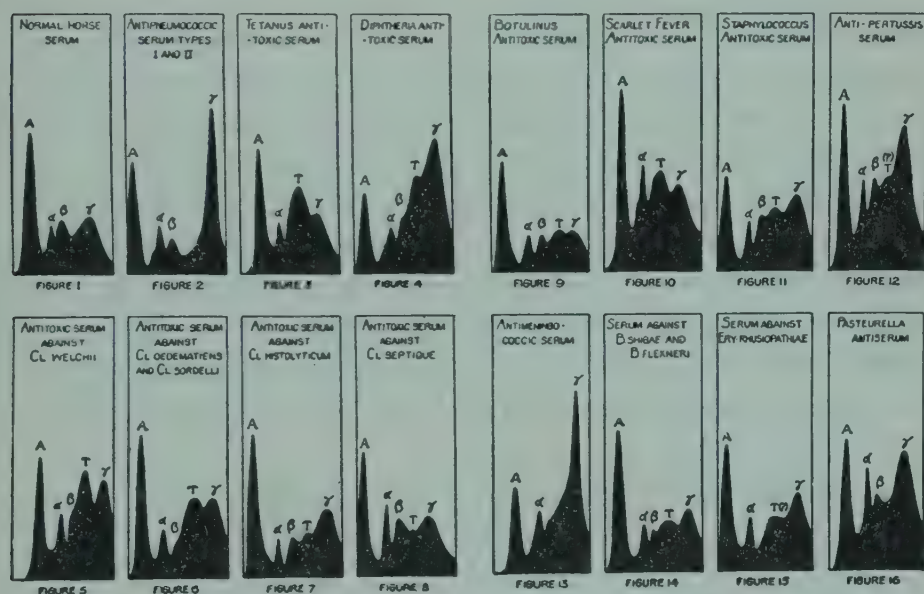
on the flocculating activity of fractions after separation by electrophoresis (9). From less definitive separations, Cooper (24) concluded that activity was associated with both beta and gamma globulins, but this would be expected if the mobility were intermediate between the beta and gamma components (see III, 25). By ultracentrifugation of syphilitic serum in the concentration cell and comparison of the flocculating activity of the supernatant and

sediment, association of the antibody with a component heavier than the gamma globulin could be established (9). Purification studies, however, (table 1) showed that only part of the antibody was of higher molecular weight (9).

The antibody activity of bovine plasma and colostrum was associated with two components, one migrating between the beta and gamma globulin and the other with the gamma globulin (24a).

The antibodies in horse antisera show much wider variations from one serum to another than do the antibodies in rabbit antisera. These variations are at least in part attributable to differences in individual response, in length and route of immunization (38), and in differences in the antigens used.

Thus, in three different laboratories (3, 25, 26), a horse anti-



—Courtesy of Williams and Wilkins Company

FIG. 14. Electrophoretic patterns of sera from normal and hyperimmunized horses. From (20).

pneumococcal serum was obtained in which the antibody was associated in the electrophoresis apparatus with a component of mobility between the beta and gamma globulins and which disappeared on removal of the antibody with specific polysaccharide (fig. 13). Numerous other sera, chiefly from horses under immunization for long periods, however, contained antibody associated with the gamma globulin. This at first led Moore, Van der Scheer and

Wyckoff (27 cf. 28) to question the original observation (3) until they themselves confirmed it (26) (fig. 13).

With other antibacterial horse sera, antibodies have been found associated with the gamma globulin (29) (fig. 14).

In antitoxic horse sera, the antibody is also associated with two components (30, 31, 32, 33). After short periods of immunization, the antitoxin appears in the gamma globulin, but on continued immunization, antitoxin appears as a component migrating between beta and gamma globulins or very close to the beta fraction. This type of antitoxin finally constitutes the bulk of the antitoxin in the serum (fig. 15). These findings are the reverse of that observed with equine antipneumococcal sera.

Electrophoretic patterns of hyperimmune horse sera are given in figure 14.

Immunological properties: In their reactivity with antigen, antibodies fall into two general categories—the precipitin-type and the antitoxin-type. The former react to give a precipitate throughout the entire region of antibody excess, whereas the latter form soluble compounds in the region of large excess of antibody as well as with excess antigen and show only a narrow zone of flocculation or precipitation. The two types of reaction curves have been considered in detail in I, 2. Only certain horse antisera are known to show the toxin-antitoxin type of reaction curve. These include the horse antitoxic sera (34, 35), equine antiovalbumin (36), antihemocyanin (37), anti-crotoxin (37a) and anti-rabbit serum al-



—Courtesy of Williams and Wilkins Company

FIG. 15. Electrophoretic diagrams on the serum of horse No. 756 taken at four bleedings during hyperimmunization against tetanal toxin. From (33).

bumin (38). The conclusion that this type of reaction is the characteristic response of the horse to immunization with protein antigens is no longer tenable since equine antibodies to rabbit globulin and to pneumococcal nucleoproteins have been found to be of the precipitin type (38, 39). Of interest in this connection are the observations that the antitoxic type of antibody is best produced in response to subcutaneous injection, whereas the precipitin type results from intravenous injection (38). In no instance have both types of response to a single antigen been produced in the horse.

Even in a single serum, antibody to a given well-defined antigen is a mixture of substances. This may be illustrated by behavior with chemically related cross-reacting antigens, where only a portion of the total antibody in the serum cross reacts. Thus in a Type VIII antipneumococcal serum, about 30 per cent of the antibody was removed by Type III specific polysaccharide and the remaining 70 per cent was strictly specific for Type VIII (40, 41) (II, 9). Similarly human and hog thyroglobulins removed 14 and 40 per cent of the total antibody respectively from an antiserum to sheep thyroglobulin, again indicating differences on the basis of ability to cross react. A detailed consideration of cross reactions is given in II, 9.

The existence of antibodies of different reactivity with antigen has been demonstrated in several ways. Methylation of the hydroxyl groups in Type III polysaccharide yielded a product which precipitated but a fraction of the total antibody from antipneumococcal horse serum, indicating that at least two types of antibody groupings were present (42).

The finding that considerably less antibody is precipitated from horse antipneumococcal sera at 37° C. than at 0° C. also indicates the existence of antibodies of different reactivity (cf. I, 2, 3). Heidelberger and Kendall (42) point out that this can not be due to differences in solubility of a homogeneous antibody—SIHI complex, since no appreciable solubility effect is observed when the reaction is carried out in a large volume.

Successive serial absorption of a serum of known antibody content with small amounts of antigen until precipitation no longer occurs does not always remove the total amount of antibody from the serum (42, 43). The antibody remaining does not alone possess the power of precipitating with antigen. If to an aliquot of this

supernatant, a fresh portion of antibody and antigen are added, the residual antibody co-precipitates with the specific precipitate and its amount may be estimated (cf. I, 2). This may be illustrated as follows: From 5.0 ml. of rabbit anti-serum albumin containing 6.60 mg. antibody N, seven absorptions with a total of 0.476 mg. serum albumin N (Sa N) removed 6.12 mg. N, after which no further precipitation occurred. On adding a 5 ml. aliquot portion of the supernatant to 1.0 ml. of antiserum and 0.215 mg. SaN, 1.788 mg. of N was precipitated as compared with 1.624 mg. N from the same amounts of antiserum and SaN alone. The difference, 0.164 mg. N calculated to the original 5.0 ml. of serum, was 0.32 mg. N not precipitated by serial addition of antigen. The recovery, 6.44 mg. antibody N (6.12 mg. precipitable + 0.32 mg. non-precipitable), was 97.6 per cent of the total antibody N (44).

This type of non-precipitable antibody has been termed "univalent" antibody, since its behavior suggested that it contained but one reactive group per antibody molecule. It has been found in the serum of horses in the early stages of immunization with egg albumin and serum albumin (45, 38). In the former instance, it was detected only by its ability to combine with specific precipitates of egg albumin and rabbit anti-egg albumin. The procedure for measuring univalent antibody is given in I, 2.

The equation for each serum (I, 2) is an average measure of behavior and reactivity of all of the different kinds of antibody to the same antigen. Changes in the equation of serum samples obtained from a given animal after each of several courses of injections have been noted. Thus the following equations were obtained in the case of a rabbit given several courses of egg albumin (43):

Course of injection	Equation calculated for 1 mg. antibody N
1	mg. antibody N pptd. = 15.8 Ea N - 62.4 (Ea N) ²
2	mg. antibody N pptd. = 20.4 Ea N - 104 (Ea N) ²
3	mg. antibody N pptd. = 24.8 Ea N - 154 (Ea N) ²

The increasing values of the intercepts 15.8, 20.4, 24.8 (2R) indicate the formation of antibodies of higher reactivity with antigen on continued immunization.

Differences in immunological reactivity of antibodies are also reflected in the findings of Goodner and Horsfall that Type I anti-pneumococcal horse sera fell into two groups with respect to their mouse protective power per milligram antibody N. One group

showed an average of 540 units and the other of 800 units per mg. antibody N. The fraction of antibody removed by small amounts of polysaccharide, i.e., that fraction with the highest combining ratio of antibody to polysaccharide, was of low protective power. No such differences in reactivity of a group of Type I rabbit anti-pneumococcal sera were found (46).

Species differences in antibodies: Differences in immunological and physicochemical properties are frequently found between antibodies to a given antigen produced in several animal species. Differences in ability of antibodies of different species to fix complement or to sensitize guinea pigs passively are apparent from I, 4 and 5. Thus far, no correlations have been found between any two properties whether physicochemical or immunological and the nature of such species differences in antibodies is as yet unexplained.

Alteration in properties of antibodies as a result of purification: While it is to be expected that changes in the properties of antibodies would result from methods of purification involving relatively drastic treatment with alkali such as in the barium or sodium hydroxide methods (IV, 43), it is rather startling to find that changes in the properties of the antibodies are found even when the salt dissociation methods are used (47) or when Felton solutions are prepared by pouring the antiserum into 20 volumes of distilled water (48)—procedures among the mildest in protein chemistry.

In purified pneumococcal antibody solutions, the amount of agglutinin N is always somewhat larger than the amount of precipitin N although in the original serum they have been found to be identical (48) (II, 11). In agreement with this, if the maximum amount of N is removed as precipitin, an additional small portion of N can be removed as agglutinin (47). In the case of one Felton solution, this residual agglutinin N was 11.5 per cent of the total antibody N (48).

No correlation between the mouse protective power of purified horse antibody solutions and their antibody content has been obtained (49), although in whole serum these two properties ran parallel (50-53).

In addition, although dissociation of specific precipitates with 15 per cent salt yields purified antibody solutions approaching 100 per cent in purity with some sera, with other sera products only 40-50 per cent pure are obtained. This is apparently a property of

the individual serum rather than due to variability of the method, since repetition with another portion of the same serum will give a solution of the same purity.

These changes do not appear to be related to changes in particle size during purification since no changes in sedimentation constant were found at any step in the salt dissociation method. Nor can they be attributed to dissociation of but a fraction of the antibody, since the equations of the purified antibodies have been found to be very close to those of the original sera from which they were obtained indicating that purified antibodies possess the same range of reactivities as they did in the native state (54).

No explanation to account for these differences in the behavior of purified antibodies has yet been offered.

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Part II

APPLICATIONS AND USES OF QUANTITATIVE IMMUNOCHEMICAL METHODS

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CHAPTER 7

ESTIMATION OF ANTIGENS

Antigens may be estimated by precipitin tests with varying degrees of precision depending on the method chosen. The qualitative methods, in which serial dilutions of antigen are added to a constant amount of serum permit estimation only of the order of magnitude of an antigen in a mixture. The results are limited by the essentially subjective method of reading the test. Greater precision may be obtained by the optimal proportions method which compares the ratios of quantities of antigen giving most rapid flocculation with antiserum. The most precise method for estimating amounts of antigen in unknown solutions (1, 2, 3) involves the use of a calibration curve prepared by analyzing a series of washed specific precipitates formed by adding known amounts of antigen to a measured volume of antiserum. These procedures have already been described (I, 2 p. 34).

The quantitative precipitin method is capable of widespread application in the analysis of mixtures containing immunologically reactive substances. It offers several very great advantages over the usual analytical chemical methods—1. It is highly specific and permits estimation of a given constituent of a mixture without chemical fractionation. 2. It requires very small amounts of material for analysis, since the amount of specific precipitate analysed is several times the amount of antigen in the sample, except for antigens of very high molecular weight. With the use of the Folin-Ciocalteau colorimetric method samples containing as little as 1 to 5 microgram antigen or antigen N per analysis are adequate. The method is capable of considerable precision, ± 2 to 5 per cent, under suitable conditions. A detailed consideration of several instances in which this technic has been applied is given below.

Estimation of specific polysaccharides: If small amounts of a serologically active product suitable for the preparation of a calibration curve with antiserum are obtainable as a result of purification, it becomes possible to estimate the amount of the substance in the crude starting material and to calculate the yield of product. This has been done for the pneumococcal polysaccharides (4) and typical data are given in table 1. To eliminate precipitin

reactions with constituents in the broth other than the Type II polysaccharide, the antiserum must first be absorbed completely with pneumococcal protein (5) and with the group-specific "C" carbohydrate (6), leaving only antibody to the type-specific substance. The calibration curve is then prepared by addition of known amounts of S II to 1.0 ml. portions of serum under a given set of conditions and the precipitates washed and analyzed in the usual manner (I, 2). Suitable dilutions of the broth are then added to 1.0 ml. portions of serum, under the same conditions and the precipitates washed and analyzed for N. From a graph of the calibration data, and the N precipitated by the broth dilutions the S II content of the dilution of broth used and the total S II content

TABLE 1

Estimation of the Polysaccharide (S II) Content of a Broth Culture and Calculation of the Yield of Purified Product

1.0 ml. Type II antipneumococcal horse serum absorbed with "C" substance and pneumococcal protein

Calibration	
mg. SII added	N pptd.
0.050	0.70
0.100	0.87
0.150	0.92

24 liters of Type II broth concentrated to 1550 ml.; 2 ml. of concentrate diluted to 10 for analysis

Volume of 1:5 dilution of concentrated broth added to 1.0 ml. serum	N pptd.	Equivalent SII content from calibration	SII per ml. 1:5 dilution of concentrate	SII per ml. of broth concentrate
ml.	mg.	mg.	mg.	mg.
0.50	0.79	0.069	0.138	average 0.134 0.670 x 1550
0.20*	0.43	0.026	0.130	
Total SII in broth				1.04 gm.
Yield on purification				0.97 gm.
Yield %				93
Polysaccharide content of broth				43 mg/liter

* Added as 0.5 ml. of a 2:5 dilution.

Heidelberger, M., and Kendall, F. E.: Unpublished data.

are calculated (table 1). The supernatants from the calibration curve and from each unknown solution analyzed must always be tested for both antibody and antigen since the method is valid only in the region of excess antibody. If supernatant tests on an unknown solution indicate the presence of excess antigen, the analysis should be repeated using a smaller volume of antigen or a higher dilution. If the unknown solution contains large amounts of inorganic salts, it is advisable to set up both calibration and unknown analyses in a large total volume of solution or to adjust the unknown solution to avoid marked differences in salt concentration. Isotonicity may be attained by dialyzing an accurately measured volume of the solution to be assayed against saline and subsequent quantitative transfer and dilution to an accurately measured larger volume.

A similar procedure may be used to determine the amount of type specific carbohydrate present in pneumococcal suspensions (7). A measured amount of the bacterial suspension is dissolved in dilute NaOH at 37° C., the solution neutralized to phenol red and centrifuged to remove insoluble debris. Care should be taken to maintain the salt concentration at about 0.9 per cent. This may be accomplished by adding to 5.0 ml. of the bacterial suspension in saline 2.6 ml. of *N* NaOH, allowing the mixture to stand at 37° C. for 72 hours, neutralizing and making the volume to 25.0 ml. with water. A known amount of specific polysaccharide should be treated with alkali under similar conditions, neutralized, diluted to 25.0 ml. and appropriate portions used to prepare the calibration curve, since in some instances alkali treatment has been found to diminish reactivity with antiserum. By this procedure two suspensions of Type I pneumococci were found to contain 0.462 and 0.493 mg. SI per mg. of bacterial N and a Type III R (Dawson "S") suspension was found to contain 0.0154 mg. SIII per mg. bacterial N. The presence of type specific substance in this strain of III R pneumococci was also established by the quantitative agglutinin method (7) (I, 3). As in the determination of the polysaccharide content of broth, absorbed sera must be used and supernatant tests must be carried out (1-3).

The same principle has been employed to determine the relative amounts of blood group A substance in various preparations and to study the pH-stability range of the blood group A substance from

hog stomachs. Preparations of blood group A substance from pools of stomachs were only 60 per cent as effective in precipitating antibody weight for weight as were similar products from individual hog stomachs showing blood group A activity (7a, 6).

Several investigators have used turbidity measurements instead of nitrogen analyses for the estimation of pneumococcal polysaccharides. A calibration curve is obtained by the addition of known amounts of antigen to a measured volume of serum and the turbidities measured. Unknown solutions are analyzed by measuring the turbidity produced when appropriate volumes are mixed with the standard volume of serum and interpolating the values on the curve (8, 9). The chief disadvantage of the method is that the turbidity readings vary with time and the rate of flocculation is affected greatly by the presence or absence of complement. The method therefore is less precise than the micro-Kjeldahl method. A new calibration curve should be prepared for each lot of serum used.

Estimation of proteins: The same methods may be used for the quantitative estimation of proteins in mixtures, provided small amounts of the substance to be measured can be obtained in relatively pure form for immunization of rabbits and for the preparation of a calibration curve. This is readily accomplished with many proteins. Table 2 shows the application of the method to determine the proportion of crystalline egg albumin N to the total N in egg white. Known amounts of crystalline egg albumin N and egg white N are added to a series of tubes containing 0.5 ml. of an antiserum to crystalline egg albumin, and the nitrogen contents of the specific precipitates, washed in the usual manner, are determined. The calibration curve is obtained by plotting total N in the washed specific precipitate against the crystalline egg albumin N added. The crystalline egg albumin content of each of the egg white samples may be determined by locating the amount of specific N precipitated on the curve and reading the corresponding amount of crystalline egg albumin N and calculating the ratio of crystalline EaN/total N added.

An alternate method of calculation consists in plotting total N precipitated against N added for the egg white data as well. The ratio of the amounts of crystalline Ea N to egg white N giving the same amount of specific precipitate N represents the proportion of

crystalline Ea N in the egg white. Thus, from a graph of the data in Table 2, 0.7 mg. specific precipitate N would result from addition of 0.024 mg. of crystalline egg albumin N or 0.073 mg. of egg white N indicating that one-third of the nitrogen in the egg white sample was crystalline egg albumin N. Table 2 shows the relative percentage of crystalline Ea N to egg white N calculated for varying amounts of specific precipitate N from 0.40 to 0.80 mg. and in-

TABLE 2

Estimation of Proportions of Crystalline Egg Albumin in Egg White

N added to 0.50 ml. anti-crystalline egg albumin	Crystalline egg albumin		Egg white*	
	Total N pptd.	Test on Supernatant	Total N pptd.	Test on Supernatant
<i>mg.</i>	<i>mg.</i>		<i>mg.</i>	
0.015	0.500	Excess A		
0.030	0.794	Excess A	0.360	Excess A
0.045	0.888	No A or Ea		
0.060	0.862	Excess Ea	0.624	Excess A
0.090			0.780	Excess A
0.12			0.866	Trace Ea
0.15			0.888	Excess Ea

Specific Precipitate N	Corresponding antigen N (from curves)		% crystalline Ea N in egg white
	Crystalline Egg Albumin	Egg White	
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
0.40	0.011	0.033	33
0.50	0.015	0.044	33
0.60	0.019	0.056	34
0.70	0.024	0.073	33
0.80	0.031	0.093	33

* Egg white not fresh.

icates the precision attainable. This method of obtaining a curve for the solution to be analyzed requires more serum and more analyses but increases the precision of the result. For most purposes,

however, determination of one or two points and interpolation on the calibration curve is adequate. Values are significant only if supernatants contain an excess of antibody.

Much smaller amounts of antigen and serum may be used by employing the Folin method (III, 22) or the Markham micro-Kjeldahl method (III, 12). Thus, following the quantitative precipitin procedure of Heidelberger and MacPherson (10), 1/10 to 1/20 the amounts of serum and antigen given in table 2 could be used. A calibration curve with these diluted reagents would have to be prepared, and the same conditions of washing of both standard and unknown solutions would have to be followed closely, since with such small amounts of specific precipitate, solubility becomes an important factor. Whenever the Folin-Ciocalteu tyrosine method is used exclusively for estimating antigens, a calibration curve of extinction against antigen N added may be used and the absolute amount of specific precipitate need not be determined.

The optimal proportions method (II) (I, 1, 2) has also been used for the estimation of the amount of crystalline egg albumin in egg white (12).

Goettsch and Kendall (2) have applied the quantitative precipitin method for the estimation of albumin and globulin in serum. Antisera to dog albumin and globulin prepared by salt fractionation were calibrated and used to determine albumin and globulin in sera, lymph, edema and ascitic fluids. In sera, results in good agreement with those obtained by Howe's salt fractionation method were obtained. These authors avoided removing the small amounts of heterologous antibody present in their antisera by calibrating both the anti-albumin and anti-globulin rabbit sera with whole dog serum of known albumin and globulin content (determined by Howe's method) and plotting the calibration curve in the usual manner based on the amount of albumin or globulin added (2).

Antisera to human albumin and globulin have also been employed for the determination of albumin and globulin in normal and pathological sera (13, 14). In nephrotic sera the amounts of albumin and globulin as measured by the quantitative precipitin method are much lower than those by the Howe method (13). Clinical improvement with disappearance of edema results in a rapid return of the globulin level determined immunochemically to normal values in agreement with those obtained by Howe's method; a longer period

was required until the albumin values obtained by both methods agreed. The discrepancy between the two methods in these sera was attributed to the presence of an abnormal globulin in nephrotic sera.

Kendall (15,16) extended the problem of estimating serum proteins by immunochemical methods by fractionating the serum globulins into three distinct antigens. Calibrated antisera to each of these could be used to estimate their amounts in various sera.

A calibrated antiserum to Bence-Jones protein prepared from urine of a patient with multiple myeloma and absorbed with normal serum may be used for the quantitative estimation of Bence-Jones protein in the serum of the same patient (17).

At present the value of immunochemical methods for the estimation of the serum proteins is limited by the fact that the albumin and globulin fractions used for the preparation of antisera, with the exception of some of Kendall's fractions (15, 16) do not consist of single well-defined proteins, but are mixtures of different antigenic substances. Individual rabbits may, therefore, react to each of the antigens in a given fraction differently and not necessarily in proportion to the relative amounts of that antigen in the mixture. Since some pathological sera may show a marked increase in one or more of the components usually found only in small amounts in normal serum, assay of these using a serum which contains little or no antibody to this component, will yield lower values by immunochemical analysis than by other methods. In these instances, it becomes difficult to establish whether the discrepancies are attributable to the presence of a new protein in the serum (13), or merely to an increase in the amount of a protein usually present only in small amounts in normal serum (16).

As a larger number of the serum proteins become obtainable in purified form, immunochemical methods for their quantitative estimation in normal and pathological sera, and other tissue fluids will become increasingly useful, since they will then provide specific and rapid methods for the quantitative estimation of each of these constituents and will require but relatively small volumes of serum. At the present time it would be of considerable importance to use immunochemical methods at least for the study of the crystalline albumin and gamma globulin fractions in normal and pathological sera, spinal fluids, etc., and to correlate these findings with electro-

phoretic patterns and salt fractionations. In this manner it would be possible to detect variations in other components by difference and to correlate such variations with clinical tests such as the cephalin flocculation and colloidal gold tests (18, 19). Immunochemical methods may also be used to study amounts of residual protein during enzymic digestion and to relate these findings to the appearance of non-protein nitrogen.

Another limitation to the use of quantitative immunochemical methods appears from the data of Bawden and Pirie (20). These authors found that tobacco mosaic virus in the leaves of infected tobacco plants did not precipitate with antiserum because it was in combination with a chromoprotein. Passage through a roller mill and tryptic digestion were required to liberate the virus from this combination. Thus, the direct estimation of the amounts of tobacco mosaic virus in leaf extracts cannot be accomplished by the quantitative precipitin method, without first removing the chromoprotein.

Immunochemical methods could readily be used to supplement or to replace less precise bioassays for estimation of proteins with biological activity. In the estimation of such substances as, for example, enzymes, protein hormones, toxins, viruses, etc., in mixtures, it is of primary importance to establish whether identical results are obtainable by quantitative immunochemical analysis and by estimation of activity. In many instances, inactivated material or precursors of these active substances may still precipitate with antiserum. In such cases use of both methods of estimation may provide evidence for the existence of such altered products.

Determination of blood volume: Quantitative immunochemical methods also provide a rapid and precise method for the estimation of blood volume. Two procedures have been employed for this purpose. Both will be described although only the second involves estimation of an antigen. Culbertson (21) injected a known amount of rabbit anti-egg albumin N into rabbits and determined the amount in a sample of plasma withdrawn 15 minutes after injection. From the concentration of anti-egg albumin in the plasma, the total amount injected, the plasma volume could be calculated as follows:

$$\text{plasma volume} = \frac{\text{mg. anti-EaN injected}}{\text{mg. anti-EaN per ml. plasma 15 minutes after injection}}$$

Knowing the hematocrit, the blood volume is obtained by dividing the plasma volume by the hematocrit.

An alternate method consists in the intravenous injection of a known amount of an antigen or of a substance giving a precipitate with an antiserum, withdrawal of a sample of plasma after allowing time for uniform distribution throughout the circulation and determining the concentration of the substance using a calibrated antiserum (22).

The former method offers the advantage in some instances that antibody homologous to the animal under investigation may be used, and also that after several weeks, the same antibody may again be used in the same animal. Large amounts of antibody are required however, for a precise estimate after dilution in the blood stream.

More accurate results may be obtained by the second method with smaller volumes of plasma, since the amount of nitrogen precipitated from a calibrated antiserum is several times the amount of antigen used. If a protein is used for determination of blood volume, the same protein cannot be used for a later determination in the same animal since antibody formation may have occurred. The use of pneumococcus type-specific polysaccharides (22) serves to eliminate this difficulty in those species in which they are not antigenic.

Qualitative methods of estimating antigens: In many instances, limited but nevertheless useful information about the amounts of material present in mixtures may be obtained by the usual types of precipitin tests. For estimation of relative amounts of antigen, decreasing amounts of antigen are added to a series of tubes containing a given volume of antiserum. The contents of the tubes are mixed, incubated at 37° C. for 2 hours and placed in the icebox over night. The amounts of precipitate are graded from — to + + + +. Light centrifugation of the tubes frequently facilitates reading. Control tubes of serum and saline, and of antigen solution and saline are included.

Table 3 illustrates the use of this procedure to follow the rate of disappearance of a substance from the blood. The material was a cotton rendered soluble by oxidation with nitrogen tetroxide (23) which, because of its content of multiple cellobiuronic acid units (II, 9), had been shown to give a precipitin reaction with Type III

and VIII antipneumococcal horse serum (24). 2.3 gms. of oxidized cotton dissolved in sodium bicarbonate were injected intravenously into a rabbit and the amount present in the circulation at varying time intervals was estimated by precipitin tests. 0.15 ml. of the rabbit serum dilutions were added to 0.15 ml. of a type VIII antipneumococcal horse serum (25).

TABLE 3

Precipitin Tests for Detecting Oxidized Cotton in the Serum of a Rabbit Following Intravenous Injection

Time Interval	Serum dilution			
	Undiluted	1:5	1:25	1:125
Before injection	—	—	—	—
2.3 gm oxidized cotton injected intravenously				
15 min.		++++	+++	++
3 hrs.	+++	+++	++	+
48 hrs.	++	+	—	—

Data from (25)

The optimal proportions method may also be used for the estimation of antigens (11). Equal volumes of varying dilutions of a standard solution and of the unknown solution are added to a series of tubes containing a given volume of an antiserum to the substance to be estimated and the dilutions giving most rapid flocculation are noted (I, 2). The ratio of the dilution factors of the unknown to that of the standard at their optima multiplied by the concentration of the standard solution gives the concentration of the unknown solution. Thus, if particulation occurred most rapidly with a 1:40 dilution of 1 per cent horse globulin and a 1:160 dilution of whole horse serum, the globulin concentration in the horse serum would be $\frac{160}{40} \times 1$ per cent or 4 per cent (11).

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CHAPTER 8

IMMUNOCHEMICAL CRITERIA OF THE HOMOGENEITY OF PROTEINS AND CARBOHYDRATES

One of the most vexing problems in biochemistry has frequently been the demonstration that a given purified material of protein or carbohydrate nature consists of a single molecular species, that is, does not contain several other contaminating substances in appreciable amounts. The usual criteria of purity used in organic chemistry, such as elementary analysis and certain physical measurements, may not be applicable or do not afford much information since different proteins (and carbohydrates) may yield almost identical results. Analysis of proteins for constituent amino acids or of polysaccharides for individual sugars may serve to establish differences or similarities between various preparations. Measurement of other physical properties such as specific optical rotation, viscosity, refractive index increment etc., may also serve to characterize such substances. The subtle nature of the differences between individual proteins or polysaccharides, however, and the difficulty of obtaining sharp separations in mixtures of these substances by chemical procedures, frequently renders the above analytical criteria of purity of limited value since they may merely be tabulations of the properties of mixtures. Even crystalline proteins have been found, in some instances, to contain impurities (I, Ia).

With substances such as hormones, enzymes, viruses, etc., showing some unique biological activity, the ratio of activity to dry weight of substance or to nitrogen content may provide evidence for the uniformity and relative purity of one preparation as compared to another. This type of assay is widely used in the purification of such materials.

Physical chemistry has in recent years contributed three noteworthy procedures, ultracentrifugation, electrophoretic analysis and solubility determination, which have been widely used in establishing homogeneity of proteins and, to a lesser extent, of carbohydrates. Each of these three procedures measures a different property and in addition effects separations of mixtures with respect to the property being measured. When used in conjunction, three independent criteria of the homogeneity of a given substance

may thus be obtained. These methods are considered in detail in Section III. Any one of these criteria alone is not sufficient to establish purity (1a) and, indeed, all three methods together may not provide complete assurance of purity.

Immunochemistry has provided several additional independent criteria (1) for the detection and, in many instances, for the quantitative estimation of impurities in protein and carbohydrate preparations.

The first of these methods involves obtaining specific antisera for suspected impurities and using these antisera to test for the impurity. If these antisera are calibrated with the antigen used to produce them, the amount of impurity in a given preparation may be estimated quantitatively by the methods outlined in the previous chapter (II, 7).

The type of contaminating substances likely to be encountered may be readily determined by a consideration of the source of the purified material. For example, broth proteins or agar frequently contaminate products obtained from bacterial cultures grown on such media and appreciable amounts of other entities derived from the micro-organisms themselves may be present. In addition purified virus preparations may contain tissue proteins.

Numerous illustrations of the use of this procedure may be given. The elimination of the group-specific "C" carbohydrate from type-specific pneumococcal polysaccharides is usually attended by considerable difficulty (IV, 51). By the use of an antipneumococcal serum from a heterologous type or from R pneumococci, the presence of "C" in a given weight of type-specific polysaccharide may be detected (II, 7). If protein is present in the type-specific substance, reactions with any antiprotein in the sera will also occur.

Contamination of products with agar may be readily detected serologically. Horse anti-sera prepared by immunization with agar-grown organisms frequently contain antibodies to agar (2-4) and give precipitin reactions with solutions of agar. Such antisera, if obtained from microorganisms unrelated to those under investigation, may also be used to detect or exclude agar in various materials. These methods are far more delicate than chemical tests for agar (5). Alexander and Heidelberger (4) found horse antiserum to agar-grown *H. influenzae* to contain 0.38 mg. anti-agar N per ml. With this antiserum, Bendich and Chargaff (6) were able to demon-

strate the absence of agar in antigens obtained from *B. Proteus*.

Immunochemical methods of detecting extraneous substances have great utility in establishing the purity of virus proteins (1). Since infected host tissues or tissue fluids provide the source for all viruses, demonstration of the absence of normal tissue constituents in purified virus preparations is of primary importance to a critical evaluation of the degree of purity and of the significance of analytical data. The problem with respect to the virus proteins is considerably more complicated than with other non-propagating substances, since the virus entity may not only be admixed with other proteins, but may also attach itself to normal tissue constituents with the production of aggregates of normal tissue protein and virus, which may show sedimentation constants or other physico-chemical properties different from those of preparations derived from non-infected tissues. In this instance, a part of the material obtained from infected tissues would be demonstrable immunologically as identical with normal tissue constituents, although the substance might satisfy the physico-chemical criteria of a single homogeneous component. These possibilities will be illustrated by specific examples.

The finding of Ledingham (7) that relatively pure elementary bodies of vaccinia and fowl pox could be prepared by differential centrifugation and of Ledingham and Gye (8) that the agent in tissue extracts which produces the Rous chicken sarcoma could be sedimented in the ultracentrifuge at 15,000 RPM and freed from a large proportion of non-viral protein, has led to the widespread use of high speed centrifugation for the concentration and purification of viruses (9-14). Products obtained from infected tissue extracts by repeated ultracentrifugation were shown to be of very complex chemical composition and to contain considerable amounts of lipid and of nucleoprotein (9).

Using these procedures, it was soon established that materials of similar complex nature could be obtained from a large number of normal tissues and organs of several animal species (15, 10). Such materials may be present in considerable amounts. For example, as much as 15 per cent of the nitrogen in clarified saline extracts of some normal tissues could be sedimented at 27,000 RPM. for one hour, as compared with 1 to 7 per cent from virus-containing tumor extracts (10). A table showing the physical and chemical properties

of materials of this type from normal and virus-infected tissues may be found in (1).

The relationship of the sedimentable constituents obtained from virus-containing tumor tissues to the products obtained from normal tissues was readily demonstrated immunologically (10). Rabbit antisera to the heavy fractions from normal chicken spleen and from chicken tumors showed identical behavior with both of these antigens in precipitin and complement fixation tests. Absorption of antisera with either constituent removed all of the precipitin and complement fixing antibody. On the basis of these findings, Kabat and Furth (10) suggested that the bulk of the material from chicken tumor tissue consisted of normal heavy tissue material and contained but a small amount of virus. The antisera to the tumor tissue were subsequently shown to contain neutralizing antibodies unrelated to the complement fixing and precipitating antibodies. The neutralizing antibodies could not be absorbed with the antigen from normal chicken spleen; antisera to the antigen from normal spleen had no neutralizing antibodies for the virus (16).

The extension of this method to other viruses with the use of quantitative immunochemical technics was indicated (1) and has now been carried out in two different laboratories using preparations of influenza virus (17, 18). Knight prepared the sedimentable protein from the allantoic and amniotic fluids of 10-14 day old chick embryos by ultracentrifugation. This normal protein showed two components in the analytical ultracentrifuge with sedimentation constants of about 80 and 170 Svedbergs, about 80 per cent of the material having the higher sedimentation constant. Both of these normal constituents were much smaller than the infectious particles from allantoic fluids infected with influenza virus which had a sedimentation constant of 600-800 S.

Knight (17a) established that the most highly purified preparations of influenza A and B viruses obtained from chicken amniotic and allantoic fluids gave precipitin reactions with antisera to ultracentrifugally purified particles from normal allantoic fluid. Likewise, samples of influenza A and B virus from infected mouse lung reacted with antisera to particles from normal mouse lung (17a,b). Values of twenty and thirty per cent by weight of normal tissue constituents in influenza A and B virus particles were obtained from quantitative precipitin curves (17a). Since the infectivity of

ultracentrifugally and electrophoretically homogenous influenza virus from chicken allantoic fluid was precipitated by antiserum to normal particles from allantoic fluid, the normal tissue antigens would appear to be an integral part of the 100 μ particles carrying virus infectivity (17a).

No differences in the content of normal antigens were found when virus preparations were centrifuged for varying lengths of time, suggesting that the normal constituent was not present in simple admixture with the virus moiety (17).

Knight also found that absorption with the virus proteins removed all of the antibody from the antiserum to the normal protein again indicating the similarity to the behavior of the chicken tumor viruses with antisera to normal chicken spleen (1, 10).

In a related study Cohen (18) prepared particles of $S = 245$ from the chorioallantoic membranes of normal chick embryos. An antiserum to these particles was calibrated with a suspension of the homologous antigen and portions were set up with preparations of influenza virus. Parallel determinations were also carried out after absorption of the Forssman antibody from the serum. Cohen reports values ranging from 27 to 57 per cent of normal antigenic material in the virus preparations.

The data of both investigators (17, 18) clearly reveal the presence of a sufficiently large proportion of normal material in the virus preparations to make chemical analyses of dubious significance, and further establish the utility of the immunochemical method as originally proposed (1, 10).

Careful consideration must be given to the selection of the normal material used for comparison with virus. For example, it is conceivable that the particles liberated into the allantoic fluid by the 10-14 day embryo (17) are more closely related to the substances with which the virus combines than are the particles from the chorioallantoic membranes (18). If this were the case, a portion of the antibody to the membrane particles might be totally unrelated to that incorporated into the virus so that interpolation of analyses would be on a calibration curve containing unrelated antibody and lower values for the amount of normal protein in the virus would be obtained. The absorption experiments (10, 17) have ruled out this possibility in case of the allantoic fluid particles.

There are other indications that the values found are minimal

and that the actual amount of normal protein in the virus preparations may be even higher. It is probable that the larger particle size of the virus materials results in a decrease in the number of reactive groupings per milligram of antigen nitrogen as compared with the smaller normal particles and hence a given weight of normal particle will precipitate more antibody N than the same quantity of virus protein.

In the case of a plant virus, tobacco mosaic, the data indicate that normal tobacco protein is present merely as a contaminant and is not incorporated into the virus protein. Beale and Lojkin (19) found by precipitin tests with antiserum to normal tobacco protein, that purified tobacco mosaic virus obtained by ultracentrifugation contained at most 1.5 per cent of normal plant protein. Antisera to the tobacco mosaic virus did not react with normal tobacco protein.

After obtaining negative tests for a suspected impurity, it is important to demonstrate that the same portion of serum can still react with the impurity itself (cf I, 2).

The second immunochemical method of obtaining evidence for the homogeneity or heterogeneity of proteins and immunologically reactive carbohydrates is based upon the results of supernatant tests with antigen and antibody at various points along the quantitative precipitin curve of the antigen-antibody system (cf. I, 2).

The principle that the supernatant of a mixture of a single antigen and its homologous antibody does not contain both antigen and antibody was recognized by some immunologists long before the development of quantitative methods made it possible to apply it as a criterion of chemical homogeneity. Thus, in 1916, Richard Weil (20) stated:

"Antigen and precipitating antibody do not coexist in the same fluid without undergoing union and precipitation. The theory that they do so coexist is fallacious, and is based on the use of a mixed antigenic substance, such as horse serum, which actually contains numerous separate antigenic substances."

Subsequent *in vivo* studies by Culbertson (21) on the role of circulating antibody in the removal of injected antigen, showed that in the case of a single antigen, crystalline egg albumin, both antigen and antibody were not simultaneously present in the circulation. With a mixture of antigens, such as whole serum, tests for

antigen and antibody could be obtained in the same sample of serum from immune rabbits.

With the elaboration of the quantitative precipitin curves obtained by addition of increasing quantities of antigen to a given volume of antiserum (cf. I, 2) it became apparent that certain antigen-antibody systems do not yield supernatants in which both antigen and antibody can be detected. Typical instances of such behavior are illustrated in tables 1, 4, 5, 6 of I, 2. In other instances, tests for both antigen and antibody in the same supernatants were obtained over a wide range. The former type of result was generally found with substances which were believed from chemical considerations to be highly purified, whereas the latter type of behavior was shown by systems known to contain several antigens.

The direct application of both the principle and the observations noted above as criteria of immunochemical homogeneity was made by Kendall (22), who studied the water soluble and insoluble eu- and pseudo-globulins from human serum and concluded that only the water soluble euglobulin (IV-38) was immunologically homogeneous because, when tested with an antiglobulin serum containing several types of antibody, it alone did not show a zone in which both antigen and antibody existed in the same supernatants (table 1). Kendall's statement defines precisely the nature of this criterion of immunochemical homogeneity:

"Positive tests for both antigen and antibody are not given by the same supernatant fluid in a system containing a single antigen and its homologous antibody. Positive tests for both constitute definite evidence that the antiserum contains more than one type of antibody and that the antigen is a mixture of antigenic components" (22).

This formulation clearly states the principle which must be used to evaluate homogeneity of any preparation and also permits previously acquired data to be critically examined with respect to their bearing on the purity of the preparation involved.

Thus, to test a given antigen for the presence of contaminants, a precipitin curve should be set up with an antiserum containing antibodies to many or all of the antigens present in the material from which the antigen was prepared. If supernatants do not show the presence of both antigen and antibody, the absence of even small amounts of these other antigens is established. For example, if a given sample of crystalline horse serum albumin gave such

results with a strong rabbit anti-horse serum, the absence of even traces of globulin in the crystalline albumin would be established.

Very few substances have been rigorously tested by this criterion. Among the serum proteins, the water soluble euglobulin from human serum and crystalline bovine serum albumin* is the only one studied which has been found to be homogeneous (table 1.)

TABLE 1

Nitrogen Precipitated from Rabbit Anti-Human Globulin Serum by Globulin Fractions

Fraction	Globulin nitrogen added	Nitrogen in precipitate	Tests upon supernatant fluids					
			Added					
			Anti-serum	Euglobulin	Euglobulin soluble	Euglobulin insoluble	Pseudo-soluble	Pseudo-insoluble
Euglobulin	<i>mgm.</i>	<i>mgm.</i>						
	0.044	0.410	—	+++			++	+++
	0.088	0.598	+	+			+	+
	0.132	0.710	+	—			—	—
Euglobulin H ₂ O soluble	0.046	0.410	—		+++±	+++±		
	0.092	0.596	—		—	±±		
	0.137	0.658	+		—	+		
Euglobulin H ₂ O insoluble	0.037	0.270	+		+++	+++		
	0.073	0.454	+		++	++		
	0.110	0.562	+		++	++		
Pseudoglobulin H ₂ O soluble	0.044	0.216	+	+++			++	+++
	0.088	0.324	+	+++			++	+++
	0.132	0.418	+	+++			+	+++
Pseudoglobulin H ₂ O insoluble	0.040	0.282	+	+++			++	++
	0.080	0.452	+	+++±			++	++
	0.120	0.556	+	++			+	+

From (22)

Of the products obtained from bacteria, two have been reported in their reaction with antisera to intact bacteria to yield supernatants which did not contain both antigen and antibody. Pennell and Huddleson (23), obtained typical quantitative precipitin curves with the endoantigens (24) (Boivin type) obtained from *Brucella abortus*, *melitensis*, and *suis* and homologous antisera prepared with intact organisms. The curves satisfied the general

* M. Mayer, unpublished observations.

type of equation (I, 2) for the precipitin reaction and supernatants showed the presence of excess antibody or antigen, or of neither component, indicating that the material was probably quite pure, provided that the sera used contained antibodies to the other *Brucella* antigens. A typical set of their data is given in table 2.

TABLE 2

Addition of Increasing Amounts of Brucella Suis Endoantigen to 1.0 ml. of Suis Antiserum

Antigen N added	Total N pptd.	Tests on Supernatant
<i>mg.</i>	<i>mg.</i>	
0.005	0.044	Excess antibody
0.010	0.079	Excess antibody
0.015	0.105	Excess antibody
0.020	0.123	Excess antibody
0.031	0.147	No antibody or antigen
0.051	0.154	Excess antigen
0.071	0.179	Excess antigen
0.102	0.191	Excess antigen
0.122	0.156	Excess antigen

Data from (23)

Values converted from four to three significant figures.

TABLE 3

Reaction of Boivin-type Antigen Obtained from E. Typhosa with 1.0 ml. of Anti-typhoid Serum

Antigen N added	Total N pptd.	Tests on Supernatants
<i>mg.</i>	<i>mg.</i>	
0.0025	0.087	Excess antibody
0.0060	0.141	Excess antibody
0.012	0.226	Excess antibody
0.025	0.312	Excess antibody
0.037	0.356	Trace antigen and antibody
0.050	0.384	Excess antigen

Data from (25)

Studies on the cross reactions between various strains of *Brucellae* are discussed in II, 9.

Hornus and Grabar (25) prepared a Boivin-type antigen by the trichloroacetic acid method (IV, 50) from the typhoid bacillus and also found their products to give typical quantitative precipitin curves with antisera to whole organisms. At most, supernatant fluids contained but traces of both antigen and antibody (table 3). It is not possible, however, to be certain that they had a pure antigen, since their sera are not definitely known to contain other antibodies. This could readily have been demonstrated by

TABLE 4

Addition of Increasing Amounts of Crystalline Horse Serum Albumin to Homologous Rabbit Antisera

Antigen N added	Total N pptd.	Tests on Supernatant	
		For Antibody	For Antigen
mg.	mg.		
1.0 ml. serum 1825 D			
0.039	0.601	++	+
0.078	1.040	++	+
0.117	1.361	++	+
0.156	1.505	++	+
0.195	1.660	++	+
0.234	1.805	++	+
0.273	1.926	++	+
0.312	2.016	+	+
0.351	2.037	+	+
0.390	2.029	+	++
0.468	1.943	—	++
0.546	1.774	—	++
1.0 ml. serum 1930 C			
0.0189	0.242	++	—
0.0378	0.392	++	—
0.0567	0.551	++	++
0.0756	0.637	++	++
0.0945	0.688	++	++
0.1135	0.761	++	++
0.1324	0.805	++	++
0.1513	0.798	++	++
0.1701	0.769	++	++
0.1891	0.725	++	++
0.2269	0.623	—	++
0.2647	0.495	—	++
0.3025	0.499	—	++

Data from (27)

Ring tests were carried out on supernatants; ++ indicates that an immediate ring was observed, and + denotes a definite precipitate on standing overnight in the refrigerator.

TABLE 5

Antibody N Precipitated from 1 ml. Type I Horse Antiserum 1095 by Varying Amounts of Type I Meningococcal Polysaccharides

Poly-saccharide added	M6C		M8CF		M8B Protamine precipitated		M9B2		18 (Scherp)		
	Anti-body N precipitated	Supernatant + M6C	Anti-body N precipitated	Supernatant + M8CF	Anti-body N precipitated*	Supernatant + M8B protamine precipitated	Anti-body N precipitated	Supernatant + M9B2	Anti-body N precipitated	Supernatant + 18	Supernatant + Anti-body
mg.	mg.		mg.		mg.		mg.		mg.		
0.02	0.248	+++	0.256	++	0.194	++	0.262	+	0.318	+	+
0.04	0.300	± (+)	0.298	++	0.251	++	0.286	+	0.406	+	+
0.06	0.332	— (—)	0.340	+	0.329	± (±)	0.312	—	0.484	+	+
0.08	0.340	— (—)	0.350	— (—)	0.340	— (—)	0.294	—	0.534	+	+
0.10	0.342	— (—)	0.350	— (—)	0.337	—	0.278	—	0.594	+	+

Readings in parentheses after centrifugation

* Corrected for additional protamine N in precipitate.

From (30)

testing supernatants for the presence of antibacterial agglutinins. Other evidence suggested that their antigen existed in several aggregates differing in particle size (25).

Several instances have been reported in which this method indicated the presence of impurities. Perhaps the best known is the case of crystalline horse serum albumin in which the presence of several antigens was established from supernatant tests. Goldsworthy and Rudd (26) reported that three times crystallized horse serum albumin prepared by the method of Adair and Robinson (IV, 37) contained as much as 2 per cent of globulin. In studies on the precipitin reaction between crystalline horse serum albumin and homologous antibody, Taylor, Adair, and Adair (27) observed a broad zone in which supernatants contained both antigen and antibody (table 4) and concluded that their serum albumin contained at least two components. Kabat and Heidelberger (28) suggested from subsequent studies that the second component was globulin and that the use of large amounts of the crystalline albumin for immunization had led to the formation of antiglobulin in addition to antialbumin.

From the behavior of supernatants in the reaction between type I specific meningococcal polysaccharide and polyvalent horse anti-meningococcal serum, Scherp (29) concluded that his products contained two components, one of which was group specific. Kabat, Kaiser, and Sikorski (30) confirmed Scherp's observations using one of his preparations and were also able to obtain products which approached immunochemical purity as determined by supernatant tests. Table 5 shows the quantitative precipitin data and supernatant tests with both the Scherp preparation and other samples with a Type I antimeningococcal horse serum.

Numerous data have been obtained with a variety of antigens and antibodies in which supernatant tests conformed to the immunochemical requirements for a homogeneous system (I, 2). These results, however, do not necessarily mean that the antigens used did not contain some impurity. In most instances, when animals are immunized with such purified substances antibodies may be formed only to the major antigen in the mixture. Under these circumstances, the system would show the behavior expected for a pure substance even if the antigen were contaminated. Such specific antisera are very valuable for estimating the amounts of homologous antigen

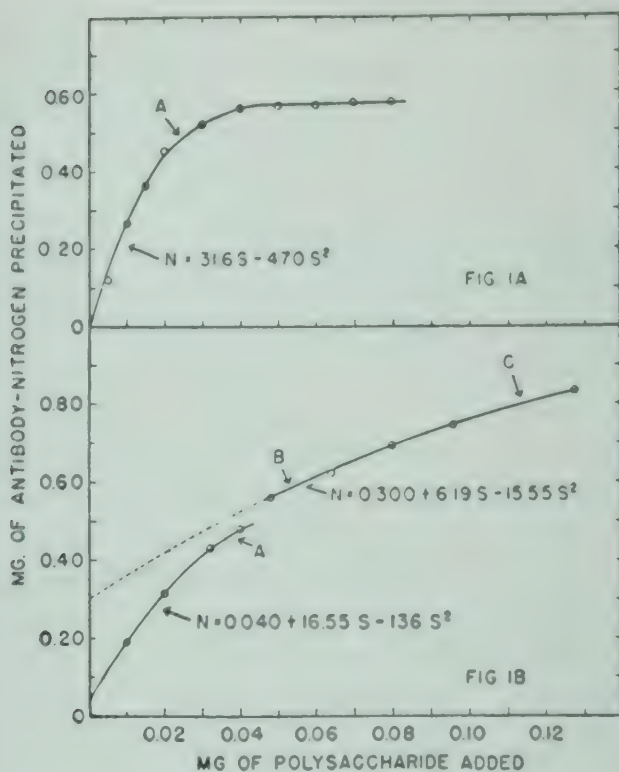
in mixtures (cf. II, 7) if they can be calibrated with a relatively pure sample of antigen. In some instances, this might permit estimation of impurities as the difference between the total N of the solution and the antigen N as determined by the quantitative precipitin method.

The use of supernatant tests as a criterion of purity is subject to certain limitations which must constantly be considered. For example, if a product has been partially degraded in the course of purification, a zone in which both antigen and antibody appear in the supernatant might be found. This type of behavior also appears to be characteristic of many types of cross-reacting systems (cf. II, 9) so that the possibility of cross-reactions should be excluded before reliance may be placed on the results of supernatant tests. Since the entire precipitin curve is usually determined in conjunction with the supernatant tests, exclusion of certain cross-reactions such as those between Types III and VIII pneumococcal carbohydrates may be made by inspection (I, 2, II, 9). It is obvious that immunologically inert substances cannot be detected by these methods and that unless the antiserum used contained antibody to any given impurity, it would not be possible to exclude the presence of that impurity.

In systems in which an antigen contains two immunologically reactive substances which have not been separated and the antiserum contains antibody to both of these constituents, Scherp (29) has devised a method for estimating the amount of antibody N to each component. The quantitative precipitin curves of such systems are not smooth like those of single antigen-antibody systems but show a definite break, at the point where one antigen first appears in the supernatant (fig. 16). Beyond this point, in the region where both antigen and antibody are found in the supernatant, the curve becomes flattened (fig. 16B). Equations computed separately for the two portions of the curve, were found to be of the following type (29):

$$\text{mg. antibody N ppted} = K + aS - bS^2 \quad [1]$$

where K is a constant and the other symbols are the same as described for single antigen-antibody systems. For some sera, K was zero for the first portion of the curve so that the equation became identical with that for single antigen-antibody reactions (31) but



Courtesy of Williams and Wilkins Company.

FIG. 16.

Fig. 16A. Course of the precipitin reaction between type I meningococcal polysaccharide and type I antimeningococcal horse serum.

Fig. 16B. Course of the precipitin reaction between type I meningococcal polysaccharide and polyvalent antimeningococcal horse serum. *From (29).*

with other sera values of 0.03 or 0.04 were found (29). For the latter part of the curve, K was rather large (0.3 to 0.5). The values of K were determined by trial and error or by the method of least squares.

If the two antigenic components are designated by S and X with S being present in greater amount and the two antibodies by anti- S and anti- X , the equation of the first portion of the curve represents the sum of the reactions ($S + \text{anti-}S$) plus ($X + \text{anti-}X$) until all of the anti- S is precipitated, as demonstrated by the appearance of S in the supernatant. The equation of the second portion covers the continued precipitation of X with anti- X . K for the second part of the curve minus K for the first part gives the amount of anti- S in the serum. Subtracting this value from the maximum precipitable N gives the anti- X content of the serum (29, 31).

The reactions of the Type I meningococcal polysaccharide preparations obtained by Scherp with an antiserum containing only anti- S and one with both anti- S and anti- X are illustrated in fig. 16.

The analytical data for the reaction with the mixed system are given in table 6.

TABLE 6

Quantitative precipitin tests with polysaccharide no. 18 and polyvalent antimeningococcal horse serum "A"

SI added	Antibody-nitrogen found	Antibody-nitrogen calculated	Difference	Supernate tested with		
				Saline	Serum	SI
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>			
0.010	0.191	0.191*	0.000	— (—)	— (—)	+++
0.020	0.316	0.316*	0.000	— (—)	± (±)	++±
0.032	0.432	0.429*	0.003	— (—)	— (±)	++±
0.040	0.482	0.482*	0.000	— (—)	— (±)	++
0.048	0.563	0.561†	0.002	— (—)	± (+±)	++
0.064	0.626	0.632†	0.006	— (—)	+ (++)	++
0.080	0.697	0.695†	0.002	—	+	++
0.096	0.750	0.751†	0.001	—	++	++
0.128	0.837	0.837†	0.000	—	++±	+
0.200	0.934					
0.400	0.931			±		—
1.00	0.894					

* Calculated from the equation, $N=0.040+16.55S-136S^2$.

† Calculated from the equation, $N=0.300+6.19S-15.55S^2$.

From (29). Courtesy of Williams and Wilkins Co.

Sera containing two such antibodies may be absorbed by adding the minimum amount of the mixed antigen to remove the anti-S completely (29, 31), as determined by the first appearance of antigen in the supernatant. Thus, if 0.048 mg. of S I were added to 1 ml. of serum (table 6), the resultant absorbed serum would be specific for X and still contain almost one-half of the total anti-X.

A method for calculating the amounts of S and X present in the antigen added, has not yet been worked out. The chief difficulty would appear to be that S and anti-S can combine in multiple proportions even beyond the point at which all the anti-S is precipitated and the composition of the specific precipitate in the second portion of the curve would be varying with respect to both S, X and anti-X.

Additional information about the degree of purity of an antigen may be obtained in some instances by the direct analysis of specific precipitates formed in the region of antibody excess for some chemical group characteristic of the antigen. Since it has been shown both by analysis (32, 33) and by supernatant tests (I, 2) that in the region of antibody excess with a pure antigen, all of the added

antigen is contained in the precipitate, the ratio with an impure preparation of the amount of antigen in the specific precipitate to the amount of substance added, gives the degree of purity of the preparation used. For example, preparations of purified blood group A substance from pooled hog stomachs contain 33 per cent of glucosamine. Analysis of specific precipitates of blood group A substance and homologous antibody (34) formed in the region of antibody excess showed that about 56 per cent of the glucosamine of the added A substance was present in the precipitate (35). The value found for glucosamine in the specific precipitate is subject to a correction for the solubility of the specific precipitate and also must be corrected for any color given by the antibody with the reagents used (35). These two factors act in opposite directions and the corrected value is not very different. Recent studies indicate that the blood group A substance obtained from pooled hog stomachs is a mixture of an active and an inactive material of very similar chemical composition. The inactive material does not precipitate with anti-A and has been identified as blood group O substance (36). With active A substance prepared from individual hog stomachs 84 per cent of the glucosamine added as A substance was found to be precipitated by excess anti-A indicating that the preparation was immunochemically pure on the basis of its glucosamine content. It is apparent, however, that the presence of impurities which do not contain glucosamine could not be excluded by this method. As would be expected, the substance from pooled hog stomachs was only about 60 per cent as effective in precipitating anti-A as were samples of blood group A substance from individual hog stomachs (37).

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CHAPTER 9

CROSS REACTIONS

While antibodies generally react only with the antigen used for immunization (the homologous antigen), certain exceptions, termed cross reactions, have been noted in which reactions occur with substances other than the homologous antigen. Although such reactions with heterologous antigens may seem to throw doubt on the general concept of immunological specificity, it has become apparent from chemical studies that cross reactions are due to structural similarities between the antigens concerned. Far from invalidating the concept of specificity, the study of cross reactions has actually contributed to an understanding of its nature. As defined here, the concept *cross reaction* applies to serological relationships between different *single* antigens, which presumably possess similar structural groupings within their molecules.

When an animal is injected with a *mixture* of antigens, distinct antibodies to each of the constituents may be found in the immune serum. If another mixture of antigens reacts with this antiserum this may be due either to a common *identical* antigen, or to a chemically related but not identical antigen. If the two mixtures contain an antigen in common, it is merely another instance of a homologous reaction, but if they contain related antigens, the system may be considered as a true cross reaction. In the investigation of mixtures of antigens it is useful to make the distinction between reactions due to a common *identical* antigen and true cross reactions. For example, all types of pneumococci contain at least two common, and presumably *identical* antigens, the C substance and the pneumococcus protein or proteins. In addition, certain types of pneumococci, like types III and VIII cross react due to a similarity in structure between their capsular polysaccharides. Failure to observe this distinction has led to much confusion in studies of soluble antigen mixtures as well as of particulate collections of antigens such as bacteria, for there has been an unfortunate tendency loosely to ascribe relationships of specificity to "common antigens" with the implication that these are identical. In other cases, relationships actually due to a common, identical antigen have been indiscriminately termed cross reactions. This difficulty should be resolved,

wherever possible, by isolation and immunochemical characterization of the antigens concerned. A number of examples in which this has been done will subsequently be discussed in detail.

In reactions between soluble or particulate antigens and their homologous immune sera it is generally found that all antibody may be removed by precipitation or agglutination with properly chosen amounts of antigen. However, when an antiserum is exhaustively absorbed with heterologous antigen, it is usually observed that only part of the antibody reacts, leaving a portion in the supernatant capable of reacting only with the homologous antigen. The extent of this heterologous reaction may be evaluated by measuring the amount of antibody reactive with the heterologous and homologous antigens, respectively, using the quantitative precipitin or agglutinin method. These absorption procedures may be used in the study of true cross reactions as well as for systems possessing the same antigen in common. Measurements made by semi-quantitative techniques, such as precipitin or agglutinin dilution titer, or optimum proportions, are often too crude to furnish the desired information, and may sometimes lead to confusing results. A case in point is the cross reaction between hen and duck ovalbumin studied by Hooker and Boyd (1) by the method of optimal proportions. It was found that the ratio of anti-hen albumin to hen albumin at optimum proportions was not affected by precipitating from the antiserum all the antibody that would react with duck albumin. When Hooker and Boyd used the quantitative method (2), however, they showed that only about 1/6 of the total antibody was removed by the duck albumin, an amount scarcely sufficient to be detectable by the optimum proportions method.

Use of the precipitin dilution titer yields even less precise results and it is indeed not an uncommon finding that removal of the cross-reactive antibody leaves the precipitin or agglutinin dilution titer for the homologous antigen unaffected (cf. 1). When it is considered that such titers are usually subject to error by a factor of two, it can readily be seen that only the removal of a major proportion of the total antibody would be detectable by such crude measurements.

Since there is only scant knowledge of the structure of native proteins, these are not well suited for studies of specificity as a function of chemical structure. The use of artificial conjugates, like the azo-proteins studied extensively by Landsteiner (3) overcomes

this difficulty, at least in part, in that different determinant groups, coupled to a protein to render them antigenic, may be compared. These studies have shown that antibodies may be obtained which are specific for the determinant group, and it has also become apparent that groups which are structurally similar can cross react and that the intensity of the heterologous reaction depends on the degree of similarity between the homologous and heterologous determinant group.

For example, antibody to *m*-amino-benzene-sulfonic acid (*m*-ABS) coupled to horse serum reacted most intensely with the homologous test determinant, viz., *m*-ABS coupled to chicken serum (chicken serum was used as protein carrier in the test antigen to avoid reaction with antibody to horse serum), but precipitation, though weaker, also occurred with a heterologous test antigen containing *o*-ABS. Using *p*-ABS coupled to chicken serum, only a very slight reaction was obtained with the antibody to the *meta* isomer. Weak precipitation also occurred with test antigens containing amino-benzene-arsonic acid or amino-benzoic acid, but in these cases only the *meta* isomers were active (4).

Cross reaction studies may be made by inhibition tests in addition to tests for precipitation with soluble antigens, or agglutination in the case of particulate antigens. The formation of a precipitate by azo-proteins and antiserum may be prevented by addition of an excess of simple compounds containing the determinant group or closely related structures. For example, the reaction between *p*-amino-benzene-arsonic acid (atoxyl) coupled to chicken serum and an immune serum to atoxyl-azo-horse serum may be inhibited by atoxyl coupled to tyrosine or by atoxyl itself, and even by arsenic acid (5). These simple substances (haptens) do not give precipitates unless coupled to a carrier protein or in some instances when coupled to resorcinol (6), but they combine with antibody to form soluble compounds and thus are capable of causing inhibition. There is generally a quantitative relationship between the degree of structural similarity among related haptens and the dilution at which they can inhibit precipitation (6).

A number of cases of cross reactions between purified, single, naturally occurring antigens have been studied by the quantitative precipitin method. In one instance, viz., the cross reaction between the type III and VIII capsular polysaccharides of the pneumococcus

(7, 8), it has been possible to correlate certain aspects of the cross-reaction with structure.

Cross reaction between types III and VIII pneumococcus polysaccharides: Antipneumococcus horse sera usually contain three types of antibody, namely, antibody to the capsular type-specific polysaccharide, anti-protein, and anti-C substance. The latter antibodies appear to be group-specific (i.e., identical antigens shared in common by all types of pneumococci) and may be measured by the quantitative agglutinin technique using a suspension of R-strain pneumococci of a type other than the one employed for producing the serum (I, 3; II, 11). The type-specific anticarbohydrate may be measured quantitatively by precipitation with a very slight excess of capsular polysaccharide.

When anti-pneumococcus type VIII horse serum is tested with the cross reacting type III capsular polysaccharide a portion of the type-specific antibody is precipitated. The same is true if type III antiserum and type VIII polysaccharide are used. When agglutinin in a type VIII serum is measured with a type III suspension, more antibody is found than can be accounted for by the group-specific antibodies, as measured with an unrelated type, for example, type I pneumococcus. This is also true in the reciprocal reaction with type III antiserum and type VIII suspension. The explanation is that the heterologous organisms combine with a portion of the type-specific antibody in addition to the group-specific antibodies. The results of a comprehensive series of studies of this system (7) in both directions, are summarized in table 1. Under the columns headed precipitin N and agglutinin N are listed the values for the maximum amount of antibody found, using the specific polysaccharide and the bacterial suspension, respectively. To estimate the group-specific antibody present, agglutinin determinations were carried out with a pneumococcus IR (Dawson S) suspension. In the last column are tabulated the sums of the agglutinin found with the heterologous type, and that measured by determination of the remaining antibody on an aliquot portion of the supernatant with a suspension of the homologous type. Since this procedure involved up to four successive sets of analyses on the same solution, agreement with the directly determined homologous agglutinin N was not always perfect.

As will be seen from the precipitin data in table 1, a larger pro-

portion of the anticarbohydrate was cross reactive in both directions in the horse sera than in the rabbit sera and that the two anti-VIII horse sera tested contained a higher proportion of cross reacting anticarbohydrate than did the anti-III horse serum. Of the rabbit sera, one type III and one type VIII serum failed to show any crossing with the heterologous specific polysaccharide, a failure

TABLE 1

Quantitative Agglutinin and Precipitin Determinations on 1 Ml. of Horse (H) and Rabbit (R) Antisera to Pneumococcus III and VIII

Serum No.	Serum type	Antipneumococcus III content		Antipneumococcus VIII content		Anti-I R	Heterologous agglutinin N + homologous agglutinin N
		Precipitin N	Agglutinin N	Precipitin N	Agglutinin N	Agglutinin N	
H607, 1:1	III	mg. 0.68	mg. 0.75	mg. 0.11	mg. 0.22	mg. 0.11	mg. 0.79
H636	VIII	0.33	0.40	0.99	1.20	0.11	1.15
H644, 2:5*	VIII	0.55	0.65	1.46	1.42	0.35	1.30
R3.50 ₂	III	2.38	2.43	0.00	0.09	0.03	
R3.49 ₂	III	0.76	0.86	0.04	0.23	0.15†	
R7.18	VIII	0.06	0.25	>1.36	1.59‡	0.10	1.56
R7.19	VIII	0.00	0.20	0.84	1.21‡	0.11	1.10
		Antipneumococcus I content					
R3.70, 1:2.5	I	0.43§	0.45				

* This serum still contained anti-C. Since the S VIII contained C substance, the relatively high precipitin N value is accounted for.

† The supernatant, now free from anti-C, gave a definite precipitate with S VIII.

‡ The supernatants from the Type III precipitin determinations were used. 0.06 was therefore added to the agglutinin found in the R 7.18 serum. Owing to the small quantities of the Type VIII rabbit sera available, analytical samples of 0.5 ml. were used. The usual error was therefore doubled.

§ The supernatant, set up with pneumococcus I S suspension, yielded no more agglutinin N. From (7)

which did not depend on the total antibody present. (Occasional type III and type VIII rabbit antisera show considerable cross-reaction with the heterologous polysaccharide.) Unfortunately most of the sera contained a small amount of group-specific antibody even after preliminary absorption with pneumococcus C substance and protein. This leads to some confusion in the interpretation of the cross reaction data obtained by agglutination. However, if group-specific agglutinin N (table 1, column 7) is subtracted from heterologous agglutinin N (table 1, columns 4 or 6) it is seen that cross-reacting anticarbohydrate as measured by the quantitative

agglutinin method approximately equals cross reacting precipitin as determined with the heterologous capsular polysaccharide.

In table 2 are given data on the addition of increasing amounts

TABLE 2
Antibody N Precipitated from Type VIII Antiserum H 644 by Varying Amounts of S VIII and S III

Amount specific polysaccharide used	Antibody N precipitated by S VIII from 1.0 ml. serum dilution	Ratio antibody N precipitated to S VIII used	Tests on supernatants	Antibody N precipitated by S III from 2.0 ml. same serum dilution	Ratio antibody N precipitated to S III used	Tests on supernatants	Antibody N precipitated by S VIII from 1.5 ml. serum dilution freed from cross reacting antibody	Ratio antibody N precipitated to S VIII used	Tests on supernatants
mg.	mg.			mg.			mg.		
0.020	0.820	16.4	Excess A*	0.400	13.6	Excess A†	0.362	18.1	Excess A
0.030				0.502	8.5	Excess A†	0.508	16.7	Excess A
0.050							0.630	12.6	Excess A
0.059				0.578	6.5	Excess A†	0.676	9.0	No A or S
0.075	1.036	13.8	Excess A*						
0.089	1.128	11.3	Trace A (?)	0.654	(5.7)	Trace S	0.678	6.8	No A or S
0.100				0.736		Excess S	0.670		Excess S
0.118	1.116	7.4	No A or S						
0.150	1.110		Excess S	0.752		Excess S	0.670		Excess S
0.177									
0.200									
0.236									
Equation:	mg. N pptd. = 21.4 S - 101 S ²						mg. N pptd. = 21.4 S - 167 S ²		
	S max. = 0.106‡						S max. = 0.064‡		
	A max. = 1.136‡						A max. = 0.685‡		

The S III-anti-S VIII reaction is inhibited by high concentrations of S III.

* A = antibody.

† In addition there was no precipitate with H 644 serum or C-absorbed pneumococcus III rabbit antiserum, showing absence of S III in supernatant.

‡ Values calculated from the equation.

From (7)

of S VIII and S III to type VIII horse antiserum, H-644. (Reaction mixtures were allowed to stand for 2 days at 0° C). Before setting up these experiments, 16 ml. of the serum were diluted to 40 ml. and absorbed with C substance, so that only precipitin for the type-specific carbohydrate would be measured. The supernatants from the cross reaction with S III were combined, treated with an excess of S III to ensure complete absorption of cross reacting antibody, and again centrifuged. Portions of the supernatant were then set up with S VIII as in table 2 in order to ascertain whether or not removal of the cross reactive antibody had resulted in a change in the quantitative relationships of the residual antibody in its reaction with the homologous antigen, S VIII. The data in table 2 are also plotted in fig. 17 after recalculation to 1.00 mg. of total antibody N to permit comparison of the different experiments (cf. I,2).

It is seen that the homologous reaction followed the equation

$$\text{mg Ab N pptd.} = 21.4 S - 101 S^2 \quad [1]$$

After removal of all cross reactive antibody (33 per cent of the total) by precipitation with S III the residual antibody reacted according to the equation

$$\text{mg Ab N pptd.} = 21.4 S - 167 S^2 \quad [2]$$

Recalculated to 1.00 mg total antibody N equations [1] and [2] (table 2) became identical, namely

$$\text{mg Ab N pptd.} = 21.4 S - 114 S^2 \quad [3]$$

showing that no fractionation had occurred with respect to reactivity with S VIII, and that the antibody removed by S III had the same quantitative relationship to S VIII as did the portion which did not react with S III.

The cross reaction between S III and type VIII antibody, however, appears to be of a different nature than the homologous reaction, and the curve (B), fig. 17, obtained by plotting the amount of antibody nitrogen precipitated against S III added, is different in form and consists of an initial steep curved portion, followed by a

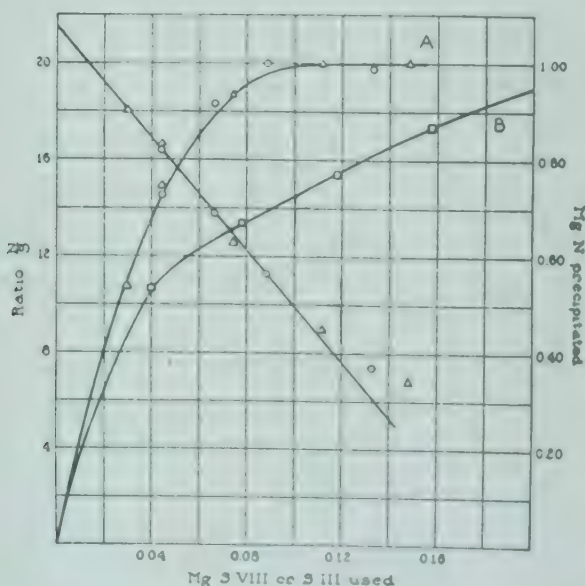


FIG. 17. Reaction of S VIII and S III in antipneumococcus VIII horse serum, absorbed with C substance.

○, S VIII-anti-S VIII reaction.

□, S III-anti-S VIII reaction.

△, S VIII-anti-S VIII reaction after removal of antibody precipitated by excess S III.

All reactions calculated to 1.00 mg. of total antibody N.

From (7).

less steep, linear section of slope 2.5. It will be noted (table 2) that a large excess of S III is required in order to precipitate all of the cross reacting antibody.

A further study (8) of the S III-anti-S VIII reaction was made with a later bleeding of horse H-644. The equation describing the homologous reaction (with S VIII) of this serum was

$$\text{mg Ab N pptd.} = 21.5 S - 108 S^2 \quad [4]$$

(total Ab N = 1.07 mg) practically the same as that of the previous bleeding taken one year earlier.

Data on the S III-anti-S VIII reaction are given in table 3 and fig. 18. The course of the cross reaction is seen to be similar to that of the earlier bleeding, with a steep initial curve, followed by a linear section of slope 2.4, practically the same as that, 2.5, of the earlier bleeding.

Two bleedings from antipneumococcus horse 909, taken two years apart, were also employed for cross reaction experiments. The data closely resembled those given by H-644. The slope of the linear portion of the curve for the earlier bleeding was 2.9 and that for the later bleeding was 2.8. The antibody-S III ratios also approached a lower limiting value as in the case of serum 644.

To separate, if possible, the antibodies characteristic of the different parts of the cross reaction plot, a quantity of SIII corresponding to a point at the beginning of the linear portion of the curve, was added to 50 ml. of serum H-644 (later bleeding). The precipitate obtained was dissociated by Liu and Wu's modification (9) of Felton's (10) dilute alkali method, and a series of precipitin analyses were carried out with the recovered antibody solutions (644-A) and S III. The curve (A, fig. 18) resembled that of the first portion of the cross reaction plot, and was described by the equation (table 3),

$$\text{mg Ab N pptd.} = 21 S - 190 S^2 \quad [5]$$

The intercept, $2R = 21$ is practically the same as that of the initial portion of the curve for 644 with S III (fig. 18) indicating that the character of the antibody in 644-A is quantitatively identical with the fraction corresponding to the initial steep portion of 644. The

TABLE 3

Comparison of Total Antibody (3.0 Ml. Serum, 1:1) and Antibody Fractions in Cross Reaction at 0°C. between S III and Anti-Pn VIII Horse Serum 644

S III added	S III in precipitate		Antibody N precipitated	Ratio anti-body N to S III in precipitate based on supernatant analyzed with H 644	Supernatants		
	By H 644*	By H 792†			+ S III	+ Anti-Pn III horse serum	+ H 644
mg.	mg.	mg.	mg.				
0.017 ₆		Total	0.344	19.5	+++		
0.031 ₃		Total	0.500	16.0			
0.047		Total	0.638	13.6	+	=	-
0.071		Total	0.706	9.9	+	=	-
0.113	0.110	0.110	0.786	7.1	-		
0.188	0.176	0.174	0.940	5.3	-		
0.245	0.220	0.213	1.04	4.7			
0.261	0.229	0.223	1.10	4.8			
0.353§	0.273	0.258	1.12	4.1			
0.423§	0.273	0.273	1.13	4.1			
0.522	0.30	0.29	1.15	3.8			

Equation for 1.00 mg Ab N: mg. Ab N pptd. = $22S - 120S^2$

Antibody N Precipitated per 6.0 Ml. of H 644 A Solution by Varying Amounts of S III

0.017 ₇		Total	0.314	17.7	+++		-
0.037 ₆ **		Total	0.514	13.7	-		-
0.062 ₆ ††		Total	0.572	9.1	-		-
0.106§	0.100		0.600	6.0	-		+

Equation: Antibody N precipitated = $21S - 190S^2$; calculated values for A at the first 3 points are 0.312, 0.521, and 0.569, respectively

For 1.00 mg. Ab N: mg. Ab N pptd. = $21S - 114S^2$

Antibody N Precipitated per 6.0 Ml. of H 644 B by Varying Amounts of S III

0.060 ₃ §§	0.057		0.334	5.9			
0.113**	0.095		0.430	4.5			
0.176††	0.128		0.495	3.9			
0.282	0.156		0.516	3.3			
0.470	0.214		0.576	2.7			

* Supernatant analyses in region of excess S III, only, carried out with anti-Pn VIII serum H 644 and quantity found deducted from amount originally taken.

† Supernatant analyses in region of excess S III, only, carried out with anti-Pn III serum H 792.

§ 2.0 ml. of serum actually used for analyses, with corresponding amount of S III.

|| Supernatants in this series were tested with H 644 A.

** 5.0 ml. of serum actually used, with corresponding amount of S III.

†† 4.0 ml. of serum actually used, with corresponding amount of S III.

§§ 7.0 ml. of serum actually used, with corresponding amount of S III.

||| 3.0 ml. of serum actually used, with corresponding amount of S III.

From (8)

supernatant from the original precipitation, designated 644-B, was also analyzed by the quantitative precipitin method. The slope of the 644-B line was 2.2, or almost the same as that of the original serum.

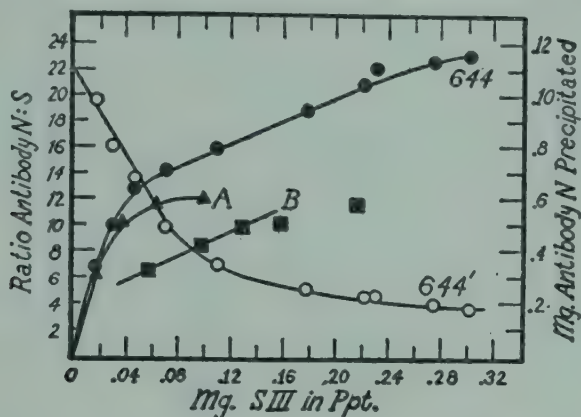


FIG. 18. Reaction of antipneumococcus Type VIII serum H 644 with S III. From (8).

It is noteworthy that removal of the 644-A antibody resulted in the failure of about one-half of the remaining cross reacting antibody to precipitate with S III. This recalls a similar effect noted in

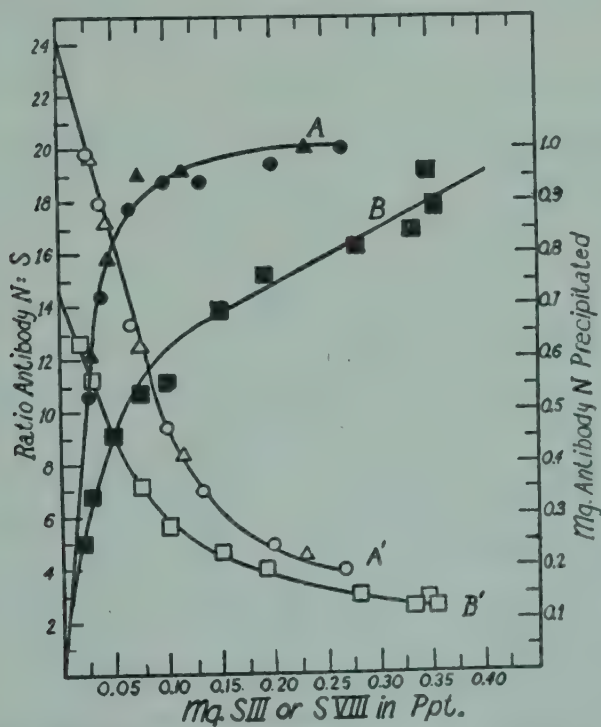


FIG. 19. Reaction of antipneumococcus Type III serum H792 with S III and S VIII. Curves A and A' are for the homologous reaction, B and B' for the cross reaction. A' and B' are the respective A N:S ratio plots. Circles give the points obtained with the original serum and triangles those after removal of antibody reactive with S VIII. Not all points in Table 4 are included in the plot.

the serial precipitation of many antisera by small amounts of antigen (I, 2, 6) and has been interpreted on the assumption that the cross-precipitable portion of the antibody is univalent with respect to antigen and can take part in aggregate formation only in the presence of sufficient multivalent antibody (11, 12). On this basis, then, the entire linear portion of the cross reaction curve would be due to the addition of univalent (and by itself non-precipitable) antibody to S III-anti-VIII aggregates, formed with the multivalent antibody of the initial segment of the curve. In the attempted separation of the antibody into portions characteristic of the two segments it would seem that sufficient multivalent antibody remained with the linear portion, 644-B, to permit precipitation, since univalent antibody alone could not precipitate with S III. The failure of line B in fig. 18 to pass through the origin also indicates that there must be an initial steep segment corresponding to multivalent antibody in 644B.

Since cross reactions are not always similar in the reciprocal sense, a quantitative study of the S VIII-anti-S III reaction was also made (8) and data on this are given in table 4 and plotted in fig. 19. The type III horse antiserum, H-792, was used after absorption with crude type I pneumococcus C substance. The precipitin determinations were carried out in duplicate in the usual way (I, 2) by addition of increasing amounts of polysaccharide to an accurately measured constant volume of serum. Reaction mixtures were allowed to stand for 2 days at 0° C.

As in the reaction with type VIII serum, the equations for the homologous reaction with S III, after recalculation to 1.00 mg. total antibody N were found to be identical before and after heterologous absorption (table 4). This may be taken to indicate a random distribution, among the antibodies of different reactivities, of groupings or configurations capable of reacting with heterologous specific polysaccharides. The course of the cross reaction (curve B, fig. 19), also, was similar to that for the antipneumococcus type VIII-S III reaction, consisting of a steep initial segment and a less steep, nearly straight portion of slope 1.1. Upon addition of larger amounts of S, the combining ratio of antibody N to S reached a lower limit showing that the precipitate attained constant composition. No inhibition ensued with 10 times the quantity of S giving maximum cross precipitation, although precipitation was delayed. A further

TABLE 4

Antibody N Precipitated from Type III Antiserum H 792 by Varying Amounts of S III and S VIII

Am't. of S used	Antibody N pre- cipitated by S III from 1.0 ml. serum	Ratio anti- body N to S III in pre- cipitate	Tests on supernatants	Antibody N pre- cipitated by S VIII from 5.0 ml. serum	Ratio anti- body N to S VIII in precipi- tate	Tests on superna- tants	Antibody N precipitated by S III from 1.5 ml. serum di- lution freed from cross reactive antibody	Ratio anti- body N precipi- tated to S III in pre- cipitate	Tests on supernatants
mg.	mg.			mg.			mg.		
0.020	0.396	19.8	Excess A*	0.250	12.5	Excess A	0.394†	19.7	Excess A
0.030	0.538	17.9	Excess A	0.336	11.2	Excess A	0.514	17.1	Excess A
0.050	0.664	13.3	Excess A	0.454	9.1	Excess A	0.620	12.4	Excess A
0.075	0.702	9.4	No A or S	0.534	7.1	Excess A	0.624	8.3	No A or S
0.10	0.700	7.0	No A or S	0.556	5.6	Excess A	0.614	6.1	Trace S
0.15	0.726	4.9§	Excess S	0.690	(4.6)	Excess A	0.654	4.5§	Excess S
0.20	0.748	3.9§	Excess S	0.756	3.9§	A and S	0.658	3.7§	Excess S
0.25				0.764		A and S			
0.30				0.816	2.9§	A and S	0.646	3.3§	Excess S
0.40				0.844†	2.5§	Excess S			
0.50				0.884	2.5§	Excess S			
0.75				0.952	2.7§	Excess S			
1.00				0.950		Excess S			
Equation : mg. N precipitated = 23.4S - 137 S ²				Equation for initial steep segment : mg. N pptd. = 14.5 S - 52.6 S ²			Equation : mg. N precipitated = 23.9 S - 143 S ²		

* A=antibody.

† Single analysis only.

§ Amount S in supernatant determined.

|| Calculated to 1.0 mg. A N as follows: in the experimentally determined linear equation,

$$\frac{A N}{S} \text{ in precipitate} = 2R - \frac{R^2}{A} \text{ S, A is put = 1.0, changing only the slope of the line.}$$

From (8)

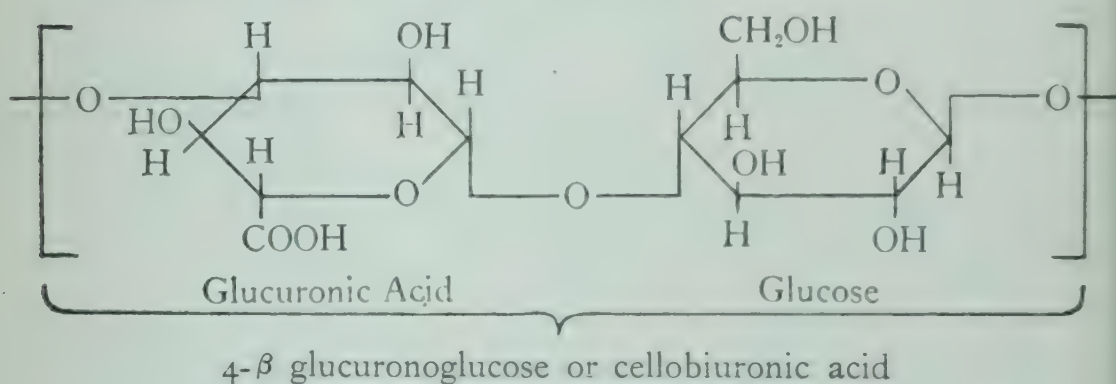
5-fold increase in S, however, resulted in nearly complete inhibition. In the reciprocal cross reaction, namely S III with anti-S VIII partial inhibition was obtained with 10 times the optimal amount of S, while a 50-fold excess of S produced complete inhibition.

In both reactions, then, soluble compounds are possible, just as in homologous precipitin reactions. The reaction between SIII and homologous horse antibody (13) for example, begins to be inhibited with about 10 times the amount of S necessary for maximum precipitation (slight antigen excess) but a further 10-fold increase is required for complete inhibition of precipitation.

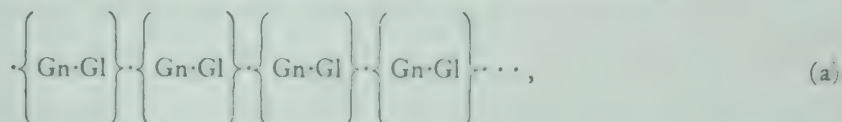
If one considers the linear segment of the cross reaction as a separate curve, apart from the remainder of the plot, it is found that the quantity of antibody precipitated is directly proportional to the amount of S precipitated, as determined from supernatant analyses. Since combining proportions of antigen and antibody remain constant over this range, it is possible to calculate an immunological equivalent weight for S in the cross reaction. The proportionality constant of antibody N to S III, or the slope, equals

2.5 for serum H-644; multiplied by 6.3 this yields a protein: polysaccharide weight ratio of about 16. If 1,000,000 be taken as the molecular weight of horse antibody to pneumococcus S (14), and its valence with respect to S III equal to 1, as discussed above, the equivalent weight of S III is found to be 62,000, or about 180 glucuronoglucose units. This would represent a minimum value for the molecular weight of S III. A similar calculation for S VIII leads to the value 140,000.

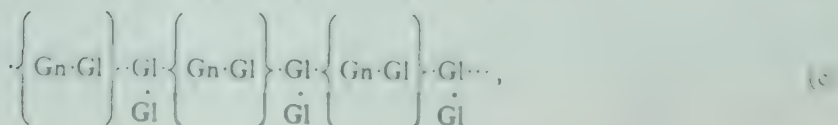
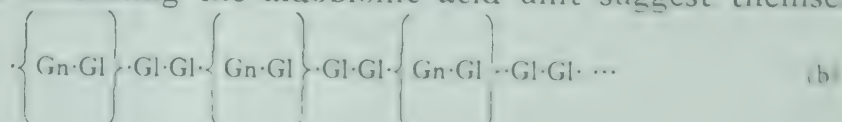
Since S III and S VIII contain the same aldobionic acid, cellobiuronic acid (glucuronoglucose), as a structural unit, cross reactivity is not unexpected. S III (15) is a poly-aldobionic acid in which the unit has the following structure:



Represented in abbreviated form, the structure is:

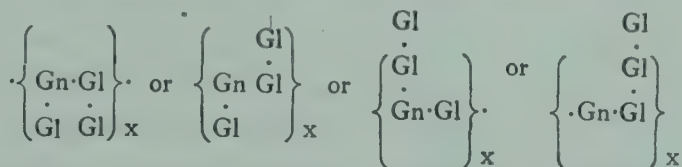


in which Gn = glucuronic acid and Gl = glucose residues, and brackets indicate cellobiuronic acid residues. S VIII (7, 16) is also a cellobiuronic acid polymer but in addition contains approximately two glucose molecules for every aldobionic acid residue. Its structure is not definitely established but a number of alternative formulae containing the aldobionic acid unit suggest themselves:





The following variations of (d) are also conceivable:



From physico-chemical and serological considerations structure (b) appears most likely. Comparison of (a) and (b) shows that in (b) every cellobiuronic acid unit would correspond spatially with alternate cellobiuronic acid units in (a). It was predicted (8) from these structures that, weight for weight, S III would be a more efficient cross precipitant for anti-S VIII than S VIII would be for anti-SIII. This would follow because in S VIII the common aldobionic acid unit, to a multiple of which the cross reactivity is ascribed, comprises only about 60 per cent of the molecule, whereas SIII is composed wholly of the polyaldobionic acid. That this prediction is borne out is shown by the slopes of the linear portion of the cross reaction curves. In the S III-anti-S VIII system these slopes are more than twice as great (2.5 compared to 1.1) as that of the single instance of the S VIII-anti-S III system studied, so that along this portion of the curve more S VIII is required to precipitate a given weight of anti-S III than is necessary in the case of SIII to precipitate the same weight of anti-S VIII. This also holds for the initial steep portion of the curves as may be readily determined from the intercepts of the antibody N:S ratio lines (figs. 18, 19).

That S III is a more efficient cross precipitant than S VIII may be further substantiated by considering the equations of the initial steep section of the cross reaction curves. For serum 644 in its reaction with S III the equation for 1.00 mg. total antibody N becomes

$$\text{mg. Ab N pptd.} = 22 \text{ S} - 120 \text{ S}^2 \quad [6]$$

The equation of the dissociated antibody (644-A) from the fractionation of serum 644 (table 3 and fig. 18, curve A) is almost the same:

$$\text{mg. Ab N pptd.} = 21 \text{ S} - 114 \text{ S}^2 \quad [7]$$

The homologous SVIII-anti-SVIII reaction of serum 644 follows the equation

$$\text{mg. Ab N ppt.} = 21.5 S - 116 S^2 \text{ (at Ab N} = 1.00) \quad [8]$$

or practically identical with that of the cross reaction over the initial steep segment.

The identity of the equations within experimental error indicates that an appreciable fraction of the antibody reacts in exactly the same way with S III and with S VIII. As far as can be determined by the sensitive quantitative method, this fraction of the antibody fails to distinguish between the two polysaccharides.

A corresponding antibody fraction with identical reactivity toward S III and S VIII was not found in the type III serum studied (table 4, and fig. 19). It will be noted that the equation of the initial segment of the cross reaction curve, calculated to 1.00 mg. of cross reacting antibody N.

$$\text{mg. Ab N pptd.} = 14.5 S - 52.6 S^2 \quad [9]$$

is distinctly different from that of the homologous reaction,

$$\text{mg. Ab N pptd.} = 23.4 S - 137 S^2 \quad [10]$$

at the same Ab N level. The failure of the two cross reactions to show strictly reciprocal behavior in this sense is in accord with the fact that S VIII is only in part a polycellobiuronic acid.

It is also evident that at least three distinct kinds of anti-capsular polysaccharide are evoked in horses in response to immunization with, for example, type VIII pneumococci. Two of these forms make up the cross reacting fraction, which usually comprises one-quarter to one-third of the total. As already noted, this portion is completely precipitable by S III or by S VIII. It may be fractionated by means of the cross reacting polysaccharide, S III, into 1) a portion characterized by a sharply ascending curve, and 2) a fraction showing a linear segment. The latter, if it could be obtained entirely separate from the other fraction, would appear to be univalent with respect to S III, although not with respect to the homologous S VIII. Finally, the principle antibody fraction, 3), at least two-thirds of the

total, is found to be rigidly type-specific in that cross precipitation between types III and VIII does not occur.

The concept "antibody" would therefore seem to refer, not to serum globulin modified in a single manner, but to a series of modified globulins separable and identifiable as distinct fractions and limited in number mainly by their cross reactivities and the quantitative criteria available for their characterization.

Cross precipitation at 37° C.: Data obtained at 37° C. on sera H-644 and H-909 (later bleeding) are given in table 5. The tubes were incubated for 0.5 hour, centrifuged at 37° C. and washed twice with saline in the cold. The supernatants were chilled and allowed to stand overnight in the icebox. Additional precipitate appeared in the supernatants which contained excess antigen. The sum of the antibody N precipitated at 37° C. and that deposited

TABLE 5

Antibody N Precipitated at 37°C. from 3.0 Ml. (1:1) Type VIII Antiserum H 644 by Varying Amounts of S III

Amount of S III added	Antibody N precipitated	Ratio antibody N to S III	Additional antibody N precipitated by subsequent chilling to 0°C.	Tests on supernatants
<i>mg.</i>	<i>mg.</i>		<i>mg.</i>	
0.020	0.250	12.5	0.003	Excess A
0.030	0.324	10.8	0.003	Excess A
0.050	0.424	8.3	0.003	Excess A
0.075	0.530		0.003	A and S
0.10	0.584		0.003	A and S
0.15	0.668		0.021	A and S
0.20	0.734		0.047	A and S
2.0 Ml. Type VIII Antiserum 909				
0.127	0.504		0.084	Excess S
0.177*	0.528		0.070	Excess S

* Three-quarters of this amount and 1.5 ml. antiserum were actually used.

From (8)

after chilling did not equal the quantity precipitated in analyses run at 0° C. throughout (cf. 17). As may be noted by comparison of tables 3 and 5, the cross reaction is subject to a marked temperature effect, similar to that observed for the homologous reaction in horse sera (1, 2). The method for preparing purified antibody by salt dissociation of specific precipitates was also found applicable to the S III-anti-S VIII cross reaction (18). For example, extraction with 15 per cent NaCl of a S III-anti S VIII specific precipitate yielded an antibody solution in which 95 and 98 per cent of the

total N was antibody N precipitable by S III and S VIII, respectively.

Oxidized cotton: It has recently been shown that cotton may be oxidized by means of nitrogen tetroxide to products containing varying percentages of carboxyl (19). These products would therefore contain cellobiuronic acid units, separated by glucose residues, at intervals in the long cellulose chain and might not unreasonably be expected to react specifically with type VIII antipneumococcus horse serum, or even with the type III antiserum.

This prediction was made by Heidelberger and Hobby (20) and actually verified with samples of oxidized cotton containing 16 to 21 per cent of COOH by weight. For precipitin tests weighed quantities of these materials were dissolved in excess N NaHCO_3 , neutralized, and diluted, first with water to approximately 0.15 N Na^+ content, and then with 0.15 N , (physiological) NaCl solution.

Qualitative tests showed that both samples, at high dilutions, precipitated antipneumococcus types III and VIII horse sera, and failed to react with type I and type II antisera.

Quantitative analyses, calculated to 1.0 ml. portions, with type VIII serum H-909, containing 1.0 mg. of type VIII anticarbohydrate N per ml. were as follows (20):

Polysaccharide added	Quantity of polysaccharide	Antibody N precipitated
	mg.	mg.
SIII	0.13	0.202
SIII	0.4	0.278
Oxidized cotton (16% COOH)	0.02	0.158
Oxidized cotton (16% COOH)	0.07	0.273

The supernatant from the last tube, plus 0.2 mg. S III yielded another 0.075 mg. Ab N cross reactive with S III, showing that roughly two-thirds of the antibody cross reactive with S III had been removed by the cotton solution. A portion of the N reactive with cotton, therefore, represents antibody non-cross reactive with the heterologous pneumococcal polysaccharide.

Analyses, calculated to 1.0 ml., with type III serum H-792, containing 0.7 mg. of type III anticarbohydrate N per ml., were as follows (20):

Polysaccharide added	Quantity of polysaccharide	Antibody N precipitated
	<i>mg.</i>	<i>mg.</i>
S VIII	0.02	0.111
S VIII	0.2	0.190
Oxidized cotton (16% COOH)	0.008	0.080
Oxidized cotton (16% COOH)	0.05	0.165
Oxidized cotton (16% COOH)	0.2	0.150

The supernatant from the last tube, plus 0.1 mg. S VIII, yielded only 0.011 of Ab N, showing that most of the antibody cross-reactive with S VIII had been removed by the cotton solution. The supernatant from the next-to-last tube gave 0.016 mg. antibody N with S VIII, while that from the 0.008-cotton tube gave 0.065 mg. The sums of the two sets of values are nearly equal to the Ab N precipitated by S VIII alone.

It is thus evident that there is a close correlation between chemical constitution and immunological specificity. Predictions as to reactivity may be made when the constitution of the repeating unit responsible for that reactivity is known.

Cross reaction between anthrax carbohydrate, pneumococcus type XIV polysaccharide and blood group A substance:—According to Ivanovics (21) the polysaccharide from the anthrax bacillus is composed of d-glucosamine, galactose and acetic acid, the latter being at least partly in the form of N-acetylglucosamine.

As shown in the table below, the same constituents have been found in S XIV, the specific capsular polysaccharide of the type XIV pneumococcus (22), and in the blood group A substance from

	Glucosamine per cent	Galactose per cent	Acetyl per cent	Other constituents
Anthrax (21)	34*	34*		nitrogenous
SXIV (22)	25		6.6–7.7	
A substance (24)	27*	32*†	9.3–10.7	amino acids

*Equimolecular proportions

† Estimated from total reducing value minus glucosamine reducing value.

hog gastric mucin (23, 24). In addition, the latter appears to contain certain amino acids (23).

As might be expected from their chemical similarity these three polysaccharides are serologically related. Data from papers by Goebel and collaborators, Finland and Curnen, and Weil and Sherman (22, 25) and by Ivanovics (26) are summarized in table 6. Ivanovics (26) used a crude preparation of S XIV made by extraction at 100° C. of washed type XIV pneumococci with *N*/10 acetic

TABLE 6
Relationships between Anthrax Polysaccharide, Blood Group A Substance and Pneumococcus SXIV

Anti-serum to	References	Species of Anti-serum	Anti-serum absorbed with	Precipitation with			Agglutination of human cells of group			Inhibition of hemolysis of sheep cells in the presence of complement				
				Anthrax polysacch.	A subst.	SXIV	Pn XIV	A	B	O	Anthrax polysacch.	A subst.	SXIV	
Anthrax	26	Horse	anthrax polysacch. SXIV PnXIV	+	+ ¹	+ ²	+	- ³						
Anthrax	26	Horse		+		-	-							
Anthrax	26	Horse		+		-	-							
Anthrax	26	Rabbit		+	-	-		-						
Human A cells	25,26	Rabbit									± ⁴	+	-	
PnXIV	25	Horse	SXIV A subst. Human cells A, B, or O		+ ⁵	+		+	+	+				
PnXIV	26	Horse			-	-		-	-	-				
PnXIV	25	Horse			-	+ ⁶		+ ⁷	+ ⁷	+ ⁷				
PnXIV	25	Horse			-	+								
PnXIV	25	Rabbit		-	- ⁸	+		-	-	-				
											Inhibition of isoagglutination of human A cells			
Human A cells	26	Human (Blood Group B)									± ⁹	+		

¹ After partial hydrolysis of A substance with *N*/10 H₂SO₄ for one-half to one hour at 100° C.

² SXIV precipitated about one-third of the antibody to anthrax polysaccharide.

³ Some sera agglutinated at low dilution, but Ivanovics (26) does not consider the reaction specific.

⁴ About 8,000 times more anthrax polysaccharide than A substance was required for inhibition of hemolysis.

⁵ Only at 0° C.

⁶ Blood Group A substance precipitated about 50 per cent of the antibody to SXIV.

⁷ Absorption of anti-PnXIV with A substance diminished but did not abolish agglutination of A, B and O cells.

⁸ Intensity of reaction greatly diminished.

⁹ About 100,000 times more anthrax polysaccharide than A substance required for inhibition of isoagglutination.

acid. Goebel's S XIV (22) was made by alcoholic precipitation (IV, 51), but the crude product was hydrolyzed with 0.14 *N* nitrous acid at 37° C. for 20 min. to destroy the C substance which was present. Both treatments (22, 26) possibly had destructive effects on S XIV and resulted in degraded products.

It was found that horse anti-anthrax sera agglutinate Pn XIV and precipitate with S XIV (26). These reactions are due to anti-

body to the anthrax polysaccharide since absorption with the latter removes all antibody reactive with S XIV. S XIV, however, reacts with only about one-third of the antibody to anthrax polysaccharide, leaving a portion precipitable only by the homologous antigen.

Blood group A substance appears to be less closely related to S XIV and to the anthrax polysaccharide than the latter are to one another. With horse anti-Pn XIV sera A substance precipitates only in the cold (25), while it reacts with horse anti-anthrax serum only after one-half to one hour's treatment with $N/10$ H_2SO_4 at $100^\circ C.$, and then only within a small range of relatively high concentrations, and in the cold (26). Hydrolysis for 2 or 4 hours abolishes the reaction. Moreover, inhibition of precipitation between S XIV and horse anti-anthrax serum takes place only with partially hydrolyzed A substance. Its inhibiting power (at 1 per cent concentration), however, increases with progressive hydrolysis, as shown by the following quantitative data (26):

Hours of hydrolysis of A substance	0	1	2	4	Control test No A substance
mg. N pptd. by anthrax polysaccharide in the pre- sence of 1 per cent A sub- stance	0.196	0.132	0.118	0.104	0.205

These remarkable observations, which recall similar findings made earlier with gum arabic (29), might be explained by assuming that the initial stages of hydrolysis expose cross reactive groups present in A substance, and that prolonged hydrolysis further degrades the molecule, rendering it unable to precipitate. As shown by inhibition tests, the degraded A substance can still combine with antibody to form soluble compounds. A similar observation was made by Landsteiner and Harte (23) who found that treatment of A substance with formamide or trypsin slightly increased its capacity to inhibit hemolysis of sheep erythrocytes but diminished its power to inhibit isoagglutination of A cells. This finding has been confirmed by Morgan and King (24).

The agglutination of human erythrocytes of all blood groups and the precipitation of A substance by anti-S XIV involves only part of the latter antibody since absorption with A, B or O cells or with

A substance leaves a portion of antibody reactive only with S XIV. It would also appear that the antibody fraction which agglutinates erythrocytes is not identical with that which precipitates A substance because absorption with A, B or O erythrocytes leaves some antibody precipitable by A substance and, conversely, absorption with A substance leaves some agglutinin for A, B or O erythrocytes. On the other hand, there would seem to be some relationship between the respective antibody fractions since each reaction is diminished in intensity by absorption with the other cross reacting antigen.

The inability of some rabbit anti-anthrax sera to cross react with A substance or SXIV, and the failure of some rabbit anti-S XIV to precipitate A substance or the anthrax polysaccharide, recalls similar differences between horse and rabbit sera in the S III and S VIII cross reaction (7, 8), and that between Friedlander type B and pneumococcus type II polysaccharides (27, 28). Rabbit antisera to Friedländer type B agglutinate type II pneumococci, precipitate S II and protect mice against infection with Pn II, and horse antisera to type II pneumococci agglutinate Friedlander type B bacilli, precipitate the type B polysaccharide and protect mice against infection with Friedlander type B bacilli. Beeson and Goebel (28) found that a number of rabbit antisera to S II did not cross react with Friedlander type B polysaccharide. Other workers have, however, found that certain rabbit sera may contain some cross reactive antibody. In the cross reaction between pneumococcus S III and S VIII it has also been observed that relatively few rabbit sera contain cross-reacting antibody.

Cross reactions of vegetable gums with antipneumococcus sera: Reactions of horse antisera to pneumococci types II and III with gum acacia were described by Heidelberger, Avery and Goebel (29). After weak hydrolysis by HCl at room temperature, which split off pentose, gum acacia formed precipitates at very much higher dilutions than did the untreated gum. Horse antisera to type II pneumococci, also have been found to react with various other vegetable gums (30). In the case of the gums studied by Mar-rack (30) treatment with HCl in the cold also extended the range of precipitation.

Analyses for maximum antibody N precipitable by various

weakly hydrolyzed gums from a horse anti-pneumococcus serum were as follows (30):

	Total N pptd. from 1 ml. horse anti-PnII serum
	<i>mg.</i>
Pneumococcus SII.....	1.55
Cherry gum.....	0.69
Acacia gum.....	0.34
Plum gum.....	0.48
Apricot gum.....	0.40
Ghatti gum.....	0.33+
Mesquite gum.....	0.33
Flax-seed mucilage.....	0.37
Tragacanth gum.....	positive qualitative test

As may be seen, the amount of antibody which cross reacted with these gums varied from about 20 to 45 per cent of the total antibody as determined by precipitation with the homologous antigen, S II.

The supernatants from the gum reactions were analyzed for remaining antibody precipitable by S II. In each case there was found to be good agreement between the sum of the nitrogen removed by the gum plus the N precipitated from the supernatant by S II, and the total nitrogen thrown down by S II from unabsorbed serum. For example, acacia gum precipitated 0.34 mg. N from 1 ml. of serum. From the supernatant 1.21 mg. N was precipitated by S II. The sum ($0.34 + 1.21 = 1.55$) is identical with the value for total anti-S II obtained by direct analysis (cf. table). If the gum had precipitated non-specific nitrogen there would not have been a corresponding reduction in the amount of anti-S II precipitated from the supernatant. Thus it was demonstrated that the protein precipitated by the gum is antibody to S II.

The supernatants after reaction with one gum were also tested with other gums. With the exception of those with flax-seed mucilage, the results indicated that the cross reacting fractions of antibody were the same for all the gums. Thus, after removal of all antibody reactive with cherry gum no reaction was obtained with

acacia. Absorption of serum with acacia, however, left some antibody precipitable by cherry gum, as might be expected from the quantitative results. Flax-seed mucilage, on the other hand, formed a precipitate after absorption with cherry gum, indicating that its reaction involves a different fraction of the antibody.

Precipitate formation by the gums was found to be inhibited by salts of glucuronic acid or of glucuronides. Galacturonic acid did not inhibit, while mannuronic acid at most delayed precipitate formation slightly. The reaction of S II was only delayed by glucuronic acid and derivatives, but quantitative analysis showed a reduction in the amount of N precipitated. As noted in (I, 2) when inhibition is not complete, quantitative analysis of the amount of specific precipitate may often reveal definite inhibition which might otherwise be overlooked.

Marrack also tested the possibility that S II is a mixture of two antigens, one of which reacts with the anti-gum portion of the antibody, and the other is specific for anti-S II. After absorption of the cross-reactive antibody from an anti-S II serum with gum, the remaining portion of the antibody was thrown down with S II. This specific precipitate was treated with hot alkali to destroy the antibody and to recover S II. When added to unabsorbed serum this recovered S II precipitated practically all the antibody showing that it could react with the cross reactive portion as well as with the homologous fraction. Thus it was shown that S II is not composed of two distinct antigens and that its cross reaction with the gums is due to structural similarity between its molecule and those of the gums. The results of Marrack's studies would seem to indicate that the uronic acids present in the gums and in S II play an important role in determining their specificity, as noted by Goebel and by Goebel and Hotchkiss in other instances (30a).

Weil-Felix reaction: The reactions between proteus OX-19, OX-2 and OX-K with various anti-rickettsial sera (e.g., epidemic and endemic typhus, Rocky Mountain spotted fever, and scrub typhus) are of great diagnostic importance. A recent investigation by Bendich and Chargaff (31) of substances extracted from proteus OX-19 with trichloroacetic acid led to the isolation of two antigens by differential centrifugation, one of which (antigen C-11) reacted only with anti-proteus sera while the other (antigen C-2) reacted with convalescent typhus sera as well. Antisera to antigen C-11

agglutinated only proteus OX-19, whereas antisera to substance C-2 agglutinated rickettsiae as well as proteus. Absorption with C-2 resulted in the removal of Weil-Felix antibodies from typhus sera.

Cross reaction between hen and duck ovalbumin (1, 2): Rabbit antisera were prepared against repeatedly crystallized hen and duck ovalbumins. From one pool of anti-hen sera 0.39 mg. Ab N per ml. was precipitated by the optimal quantity of hen-ovalbumin, but only 0.067 mg. by the optimal quantity of duck-ovalbumin; with a different pool the corresponding figures were 0.49 and 0.076 (2). Therefore, only 16 to 17 per cent of the anti-hen was precipitable by the duck antigen.

More extensive data were obtained by the dilution titer and optimal proportions methods (1). Thus, early bleedings from rabbits immunized with duck ovalbumin contained no anti-hen antibody. One anti-hen serum (R-619), however, reacted to a dilution of 10^{-3} with duck ovalbumin, while the homologous hen antigen precipitated to a dilution of 10^{-4} . In later bleedings, a pool of two anti-duck sera gave precipitates up to a dilution of 10^{-4} with both antigens, as did a pool of two anti-hen sera.

Absorption of anti-hen or anti-duck sera with the heterologous antigen did not appreciably affect the dilution titer for the homologous antigen. For example, anti-hen serum, R-619 (1) at dilutions of 10^{-3} , 10^{-4} and 10^{-5} gave +, +, 0 reactions with hen ovalbumin. After removal of the cross-reacting portion of the antibody by absorption with duck-ovalbumin, it gave +, \pm , and 0 reactions at the same dilutions.

Tests by the optimum proportions method also failed to reveal a diminution of antibody to the homologous antigen after absorption with heterologous protein. Anti-hen serum R-592 gave an optimum proportion ratio of 750 (magnitude of most rapidly flocculating antigen dilution divided by the serum dilution, that is, $7500/10$ in this case), and of 735 before and after absorption with duck-ovalbumin, respectively. These values are identical within the experimental error of the method.

It is evident then, that the absolute, quantitative method, when applicable, is superior in terms of sensitivity and precision, and that it yields information which semi-quantitative techniques do not reveal.

Since the antigen preparations employed by Hooker and Boyd

(1, 2) constitute single chemical substances and were shown to be highly pure, their cross reactivity must be due to structural similarities, and shows that there are different groups in an individual protein which give rise to various antibodies of different reactivities. This is quite analogous to the case of the cross reaction between S III and S VIII.

Cross reaction between the thyroglobulins of different species (32): Rabbits were immunized with suspensions of alum-precipitated human, hog, beef and sheep thyroglobulins (Tg). Owing to the presence of small amounts of serum globulin in the Tg preparations all anti-Tg sera were absorbed with small quantities of serum globulin of the same species as the Tg used for injection until no further precipitation occurred. Only after this had been

TABLE 7

Approximate Percentage of Cross Reacting Antibody in Anti-thyroglobulin Rabbit Sera

Serum No.	Amount of cross reactive antibody per cent	Serum No.	Amount of cross reactive antibody per cent
Hog-anti-human		Beef-anti-sheep	
3.56 ₁	20	4.93 ₁	80
3.99 ₁	20	4.93 ₂	80
3.99 ₂	36	Hog-anti-beef	
3.99 ₃	40	4.84 ₁	(50)
Human-anti-hog		Beef-anti-hog	
CQ4 ₂	15	4.94 ₁	45
CQ5 ₁	20	Human-anti-sheep	
2.61 ₃	30	4.93 ₂	14
2.64 ₁	18	Hog-anti-sheep	
3.75 ₁	12	4.93 ₂	40
3.75 ₂	21	Human-anti-beef	
Sheep-anti-beef		4.84 ₁	15
4.84 ₁	(75)	4.83 ₁	15

Values in parentheses are approximate.
Subscripts to serum numbers indicate the course of immunization.
From (32)

done was the Tg-reaction studied. With regard to the proportion of antibodies to serum globulin found after three courses of immunization, one anti-hog Tg rabbit serum contained 0.07 mg. of anti-serum globulin N and 1.07 mg. anti-Tg N per ml. Presence of excess serum globulin did not affect the amount of N precipitated by Tg from its antisera. No immunological relation between serum globulin and thyroglobulin could be detected, in agreement with Hektoen and collaborators (33).

Hektoen et al (33) showed that mammalian thyroglobulins were closely enough related to be termed organ-specific, but that they were not necessarily identical. This conclusion was confirmed and extended by Stokinger and Heidelberger (32). In table 7, data are given on the relative amount of cross reacting antibody in various anti-Tg sera. It will be noted that the proportion increases, in general, with prolonged immunization, as would be expected. Moreover, the reciprocal cross reactions appear to occur to about the same extent. The relation between sheep and bovine Tg appears to be the closest of those studied, since about 75 to 80 per cent crossing was observed. According to Adant and Spehl (34) sheep and bovine Tg show little crossing, but it is difficult to say why their findings disagree with those in (32). Human Tg differs markedly from the others in the series, for in most sera obtained after short courses of immunization, crossing was less than 20 per cent in either direction.

It was also shown in (32) that the Tg cross reactions may be quantitatively expressed by equations of the type,

$$\text{mg. Ab N pptd.} = 2RTg - \frac{R^2}{A} Tg^2$$

which have been shown to be applicable to many homologous precipitin systems (cf. I, 2). In this respect the Tg cross reactions differ from the S III-S VIII cross reactions.

It was found (32) that $2R$ is usually considerably lower in the heterologous reactions than in their homologous counterparts. Since the molecular weights of human Tg and hog Tg, at least, have been shown to be the same, namely 700,000 (35), the difference in ratio may be most simply explained by the assumption that antibody (in excess) reacts with fewer immunologically active groupings on the heterologous Tg molecule than on the homologous

Tg. The number of reactive groupings in or on the large thyroglobulin molecule must be very large since the range of combining proportions with antigen is so great. Values for 2 R in the homologous Tg systems do not, ordinarily, exceed 8. If the ratio of molecular weights of antibody to Tg be taken approximately as 150,000:700,000, or 0.21, the Ab:Tg ratio of 8 would correspond roughly to the empirical composition TgAb_{40} . The equivalence zone ratios of 3 and 2 would then be roughly equivalent to TgAb_{14} and TgAb_{10} , while the ratio 0.4 in the region of excess antigen would indicate an empirical composition of about TgAb_1 .

In agreement with Snapper and Grünbaum (36) no inhibition of Tg-anti-Tg precipitation by diiodotyrosine or by thyroxine was observed. The conclusion drawn in (36) that these two substances do not exist as such in Tg may, however, not be justified. A simple calculation shows that even the large Tg molecule contains at most two or three thyroxine groups, not more than eight to twelve diiodotyrosine units, and much unsubstituted tyrosine as well (37). Since Tg contains possibly 40 or more immunologically reactive groupings there is no reason to consider the few iodinated amino acids as necessarily significant to serological specificity. Indeed, the failure to effect a change in the reactivity of serum albumin with antisera to serum albumin by introduction of large arylazo groups into at least a high proportion of the tyrosine residues present in the antigen (38) indicates that chemical changes in the tyrosine groupings are not necessarily accompanied by pronounced changes in specificity.

Brucella endoantigen system: Agglutination studies on smooth strains of *Brucella abortus* (BcA), *Brucella suis* (BcS) and *Brucella melitensis* (BcM) by Wilson and Miles (39) showed that all the agglutinins could be absorbed by the homologous or heterologous organism, but that much larger quantities of heterologous organisms were required. These authors therefore postulated the existence of two antigens A and M which were present in varying amounts in the three varieties of *Brucella*. The studies of Miles (40) in which the optimal proportions technique was used, also supported this hypothesis.

Several groups of workers have studied *Brucellae* chemically (41-46) and some have attempted to isolate the postulated A and M antigens, but as yet none has succeeded. All purified antigens

from *Brucellae* contained both factors. Antigenic and toxic materials have been prepared from the brucella group by extraction with trichloroacetic acid using Boivin's methods (47, 48) (cf. IV, 50). These preparations resembled those obtained from other organisms, and consisted of phospholipid-polysaccharide-protein complexes (49, 50), and materials obtained from *melitensis*, *abortus* and *suis* were all found to cross react. Pennell and Huddleson (51) have studied the quantitative precipitin reactions of these antigens with homologous and heterologous goat antisera prepared with suspensions of *Brucella*. Data on the reactions of *suis*, *abortus* and *melitensis* antisera with each of the three antigens are plotted in figs. 20, 21, and 22, respectively.

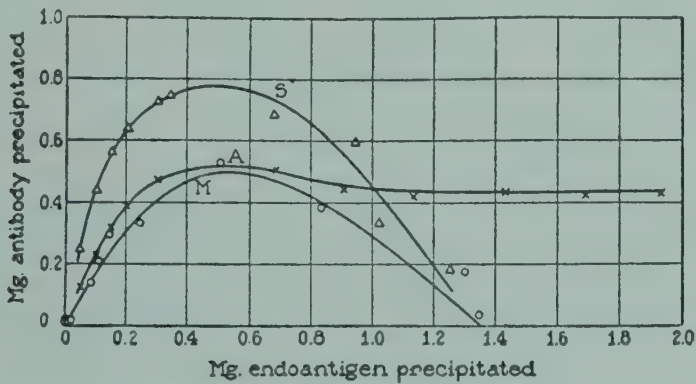


FIG. 20. *Suis* serum. A, *abortus* endoantigen; S, *suis* endoantigen; M, *melitensis* endoantigen. From (51).

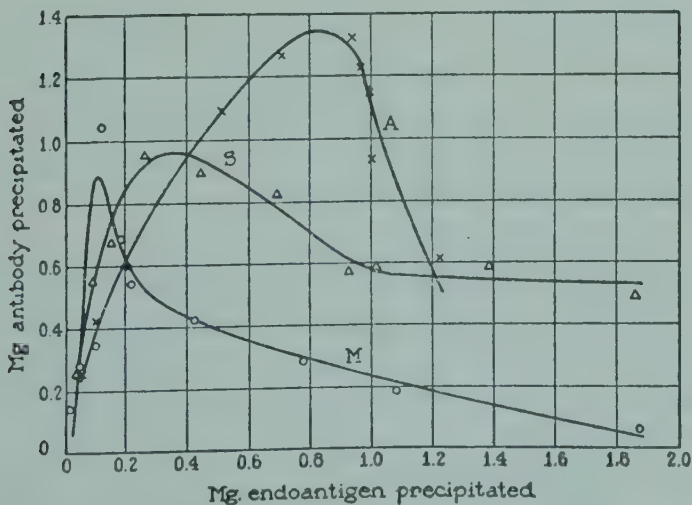


FIG. 21. *Abortus* serum. A, *abortus* endoantigen; S, *suis* endoantigen; M, *melitensis* endoantigen. From (51).

While it was found that the homologous reactions behaved as single antigen-antibody systems (cf. II, 8) (supernatants showed

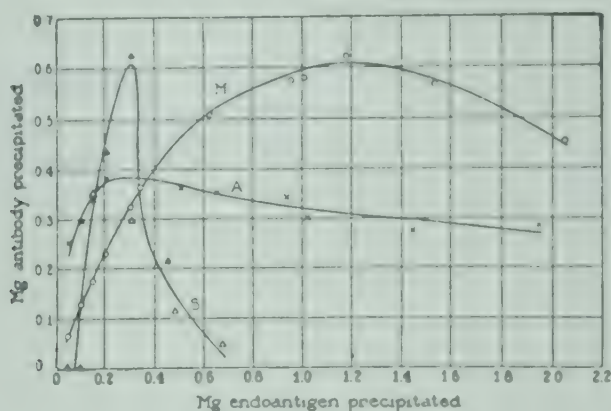


FIG. 22. *Melitensis* serum. A, *abortus* endoantigen; S, *suis* endoantigen; M, *melitensis* endoantigen. From (51).

either antigen or antibody but not both in excess), the cross reactions were entirely different in this respect.

Suis antiserum in its reaction with *abortus* endoantigen (BcA) showed a well defined region of antibody excess, an equivalence zone in which the supernatants contained neither excess of antigen or antibody, and an antigen excess zone. In its reaction with BcM, *suis* antiserum showed antigen excess in all supernatants even in those in the region in which antibody was only partially precipitated.

Abortus antiserum exhibited a similar pattern of supernatant tests both with BcS and BcM in which antigen and antibody co-existed over a wide range. Antiserum to *melitensis* in its reaction with BcA and BcS, displayed distinct zones in respect to supernatant tests like those typical of homologous reactions.

The quantitative course of the cross reactions was, however, found to differ markedly from the behavior of the homologous systems, as may be seen in figs. 20, 21 and 22. From *suis* and *abortus* antiserum the cross reacting antigen precipitated about two-thirds of the total antibody, while from *melitensis* antiserum all of the antibody could be removed with BcS. This reaction was unusual in that it took much less BcS than BcM to precipitate all of the antibody. This was explained by assuming *melitensis* endoantigen to contain a grouping which occurs much more abundantly in *suis* endoantigen, causing the latter to unite with more *melitensis* antibody per unit weight of BcS than the homologous antigen BcM

(51). BcA, however, reacted only with about 60 per cent of the total antibody.

Since there was almost no inhibition in the BcA-*suis* antiserum system it would seem that BcA can enter into fewer combinations with *suis* antibody than can the homologous BcS antigen. In accord with this were the lower combining ratios of BcA with *suis* antibody than with *abortus* antibody, showing that BcA contains fewer reactive groups for *suis* antiserum than for *abortus* antiserum. The presence of BcS in all supernatants of the BcS-*abortus* antiserum system suggested that the combination of antigen with antibody in this reaction is loose, that is, dissociation is appreciable. The same appeared to be true for the BcM-*suis* antibody system.

While the comparative relationships depicted in Figs. 20, 21, and 22 were found to be the same in antibody from different goats, the curves from different sera were never identical. To identify an unknown brucella goat antiserum it appears to be necessary to determine at least two points of the curve in the region of excess antibody with each of the three endoantigens, and from these points, to establish the equation and to sketch the respective curves (51).

To type an unknown organism, it would be necessary after preparing the endoantigen to perform two precipitations in duplicate with a calibrated antiserum, preferably *abortus* (51).

The interpretation of these antigenic relations in terms of the chemical structure is not yet possible, but it is evident that while these antigens are closely related to one another they are serologically distinct as revealed by quantitative precipitation studies with specific goat antisera.

It is noteworthy that some cross reacting antigens can precipitate all of the antibody from an immune serum and can sometimes do it more efficiently (i.e., requiring less antigen) than the homologous antigen. When there is complete precipitation of antibody by a heterologous antigen it is not possible to prepare type-specific sera by cross-absorption. It is, indeed very difficult, or even impossible, in such cases, to distinguish heterologous from homologous antigen by qualitative tests.

The quantitative studies of Pennell and Huddleson (51) strongly suggest that the Wilson and Miles hypothesis (39) of two chemically identical antigens which vary in the amounts present in different varieties of *Brucella* is an oversimplification, and that *abortus*, *suis*

and *melitensis* contain closely related but not identical antigens, which cross react to a very considerable extent. If the Wilson and Miles hypothesis were correct, the cross reaction with purified heterologous antigens should have shown the same reaction curves as did the homologous antigens and precipitate as much antibody nitrogen as the homologous antigens. Such was not the case, however, and it does not seem necessary to postulate the two antigens A and M. The *brucella* system is perhaps described best in terms of closely related chemical units present in the three varieties of endoantigens.

Plant viruses: Stanley and Knight (52) made a serological study of tobacco mosaic, yellow aucuba, green aucuba, Holmes' masked, Holmes' ribgrass, J14 D₁, and cucumber viruses 3 and 4, using qualitative precipitin tests with antiserum to tobacco mosaic virus. All of these viruses gave strong cross reactions (to antigen dilution of 1/256) with the exception of Holmes' ribgrass, and cucumber viruses 3 and 4, which precipitated only to a dilution of 1/64. The endpoint of the homologous tobacco mosaic virus reaction was about 1/1024. These results indicate a close serological relationship among these plant viruses. Analyses for aromatic amino acids (tyrosine, tryptophane and phenylalanine) were very close for all except Holmes' ribgrass and cucumber 3 and 4, which showed pronounced differences from those of the others in content of individual aromatic amino acids. Thus, there appears to be a similarity in the chemical and serological characterizations.

Bacterial cross reactions: Quantitative data on the agglutination reactions of *Neisseriae* with homologous and heterologous antisera are given in table 4 of I, 3. Extensive cross reaction was observed between *N. gonorrhoeae* and *N. intracellularis* while much less relationship appeared to exist between *N. sicca* and *N. catarrhalis* (53). It cannot be stated whether the heterologous reactions are due to the presence of one or more common identical antigens in the different varieties of *Neisseria* or due to cross reacting antigens.

In summary it may be stated that cross reactions generally show different quantitative behavior from homologous reactions and the characteristics of different cross reacting systems vary greatly. Moreover, reciprocal cross reacting systems are not necessarily alike.

It appears likely that further study of cross reactions will reveal more information about the nature of serological specificity. The studies of Pauling (6) with simple compounds containing multiple reactive groups represent a step in that direction, that is, the comparison of compounds of known structure. Unfortunately the structures assigned to these antigens are based on their formula weight rather than on their molecular weight as determined by physicochemical methods. Since azo-dyes like those employed by Pauling tend to form aggregates, the chemical formula may not represent the structure of the actual unit as it exists in solution. Furthermore, aggregation of two bivalent molecules could yield a tetravalent unit so that, if aggregation occurred, both valence and structure would be unknown. Boyd and Behnke's (54) finding that their compound "VII", an arsanilic-phloroglucinol derivative, (also used by Pauling) was aggregated to the extent of 10 to 12 molecules per particle, would make it appear as distinctly possible that some of Pauling's conclusions may require revision.

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CHAPTER 10

EFFECTS OF CHEMICAL TREATMENT IN ALTERING IMMUNOCHEMICAL PROPERTIES OF PROTEINS AND POLYSACCHARIDES

Some relationships between structure and serological specificity have been traced in the chapter on cross reactions of naturally occurring antigens. The present chapter deals with another aspect of this relationship—alterations in the specificity of antigens resulting from artificially induced structural changes.

The various kinds of chemical treatment which have been studied for their effect on the specificity of proteins may be conveniently classified into two broad groups: 1) those which involve conjugation, and 2) those which merely alter the structure of the protein without attaching foreign groups. As shown by Landsteiner's studies (1) on artificially conjugated proteins, the introduction of foreign groups generally confers upon the protein specificity characteristics typical of the foreign radical. While these investigations focused attention on the specificity of the conjugate groups and were not concerned with other effects on the carrier protein, they played a dominant role in elucidating the relation between chemical structure and specificity.

For example, Landsteiner and van der Scheer (2) prepared antisera to horse serum coupled with amino-benzoylated dipeptides, namely, glycyl-glycine, glycyl-d,l-leucine, d,l-leucyl-glycine and d,l-leucyl-d,l-leucine. Test antigens were made by coupling the amino-benzoyl derivatives of these dipeptides to chicken or ox serum. The four antigens were found to differ serologically, their specificity depending largely on the structure of the terminal amino acid carrying the free carboxyl group, and to a lesser degree also on the second amino acid. Later experiments with antisera for tri- and pentapeptides (of glycine and leucine) (3) showed that cross reactions generally occurred with peptides having the same terminal amino acid. Other portions of the molecule, however, played a role and the degree of cross reaction was definitely associated with similarities in constitution.

Similar studies showed that antibodies could distinguish between the steric isomers, dextro, levo and meso-tartaric acid (4). The

serological differentiation of stereo-isomers of sugars was investigated by Avery and Goebel (5), who obtained antibodies which were specific for glucose or galactose, two isomeric hexoses, and could distinguish them. (Cf. also studies on disaccharides (6), uronic acids (7), and aldobionic acids (8).)

In these and related studies it was noted (1) that the artificial conjugation of a protein often diminished its reactivity with antisera to the native carrier protein and it became evident that changes may occur other than the introduction of the foreign specificity. Quantitative studies of this effect have been made in a number of cases.

Azo-dye-egg albumin system (9, 10, 11): R-salt-azobiphenylazo egg albumin (DEa) prepared as described in IV-44, can be fractionated with sodium sulfate into products of various solubilities, the least soluble containing the most dye, and showing the least cross reaction with anti-egg albumin (anti-Ea). The product used in (10) was fractionated as described in (9) until it reacted only very slightly with some anti-Ea sera. Rabbit antisera to this dye, however, contained antibody reactive with egg albumin (Ea) indicating that the original specificity had not been entirely abolished.

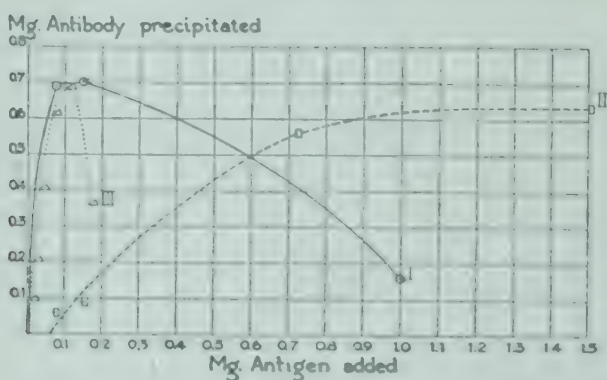


FIG. 23. Curve I DEa-Anti-DEa reaction.
 II Ea-Anti-DEa reaction.
 III Ea-Anti-Ea reaction.
 From (10).

Data on the DEa-anti-DEa and Ea-anti-DEa reactions are listed in table I and plotted in Fig. 23. For comparison, a reaction curve of Ea with anti-Ea of similar precipitin content is given (curve III, fig. 23) (10).

In the homologous reaction, DEa with anti-DEa, supernatants

TABLE 1

Precipitin Reactions of Dye-Ea and Ea with 1 ml. Rabbit Anti-DEa Serum

Antigen added	Total protein precipitated	Antibody protein precipitated	Reactions of supernatants with:
mg.	mg.	mg.	
dye-Ea			anti-dye-Ea
0.075	0.75	0.68	—
0.15	0.78	0.70	+
1.0	0.19	0.16	
Ea			anti-Ea
0.075	0.07		++
0.15	0.10		+++
0.72	0.61		++++
1.50	0.69		

Data from (10)

in the region of antibody excess contained no free DEa, as is also the case in the reaction of Ea with anti-Ea. From curves I and III, it may be seen that the two homologous reaction systems follow a similar course except that the DEa-anti-DEa reaction is less readily inhibited.

In the cross reaction between Ea and anti-DEa, excess Ea was present in all supernatants even in the region of excess antibody, showing that the antigen-antibody union in this case is a highly dissociated one. The course of the reaction curve (II in fig. 23) also indicated that this reaction is of a different character than either of the homologous reactions. It required much larger amounts of Ea than of DEa to reach maximum precipitation, and the reaction was not inhibited even by very large amounts of Ea, indicating that Ea cannot form soluble compounds with anti-DEa.

In anti-DEa sera other than the one given in table I and fig. 23 results were similar, except that in some cases a smaller fraction of the total anti-DEa was precipitable by Ea. Serum 8.01 (10) for example, contained 1.94 mg. of anti-DEa per ml., but of this only 1.09 mg. could be precipitated with Ea. The supernatant from the latter analysis yielded practically all of the remaining antibody on addition of DEa. Although there is no *a priori* reason why the

DEa antigen might not be split in the animal body so as to give rise to the same antibody as does Ea itself, the differences in properties of anti-DEa and anti-Ea indicate that this does not occur. For example, the Ea-anti-Ea and Ea-anti-DEa reactions are very different in their quantitative aspects. In addition, the observation that the sum of successive Ea-anti-DEa and DEa-anti-DEa precipitations equals the total DEa-anti-DEa and the fact that free antigen may be detected in supernatants from precipitations in the antibody excess zone of the Ea-anti-DEa reaction, but not in those from DEa-anti-DEa interaction support the conclusion that anti-DEa and not anti-Ea is formed on immunization with DEa.

The changes produced in Ea by coupling it with dye are probably such that antibody is formed to certain antigenic groups characteristic of native Ea as well as to new configurations formed as a result of introducing dye groups. This would follow from the observation that Ea behaves like a cross reacting antigen in its reaction with anti-DEa while the DEa-anti-DEa reaction shows behavior characteristic of a homologous antigen-antibody system.

R-salt-azobiphenylazo-serum albumin (DSa): This protein derivative containing about 15 to 16 dye groups per molecule of serum albumin (Sa) was prepared from crystalline horse serum albumin according to (12) as described in IV, 44. In contrast to the egg albumin-dye, DSa reacted as strongly with anti-Sa as did Sa, and attempts to obtain fractions free from reactivity with anti-Sa proved unsuccessful (12). Indeed, a series of quantitative precipitin determinations of the reaction between anti-Sa serum and varying amounts of DSa yielded a reaction curve identical with that given by Sa (fig. 24).

However, in the reciprocal cross reaction, Sa removed only part of the antibody against DSa, and additional antibody was precipitated by the homologous antigen, DSa. With anti-DSa serum 4.35, for example, about 0.35 mg. antibody N was precipitated by Sa. On addition of DSa in optimal quantity to the supernatant another 0.23 mg. antibody N was obtained, bringing the total recovery of antibody to about 0.58 mg. N. Direct analysis with DSa, however, yielded 0.73 mg. total antibody N, so that about 0.15 mg. N had failed to precipitate in successive analyses with Sa and DSa. Sixty per cent of this residual antibody could be recovered by adding a portion of the supernatant to a mixture of DSa and

unabsorbed serum 4.35, but no additional antibody was taken out of another portion by a mixture of Sa and anti-Sa, showing that the residual univalent antibody (I, 2, 6) contained anti-dye groupings rather than anti-Sa groupings.

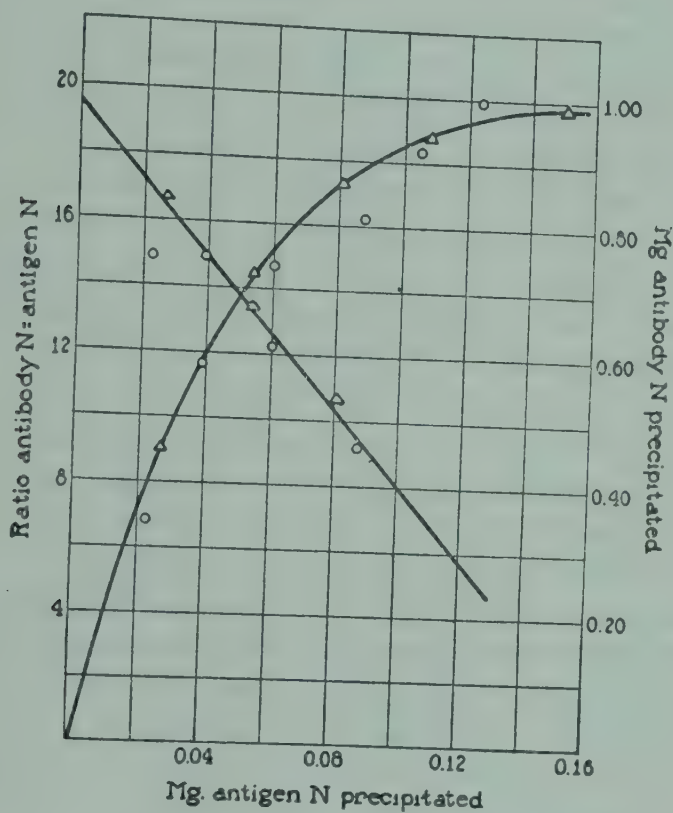


FIG. 24. Precipitation of anti-Sa by DSa and Sa.
 O, DSa-anti-Sa reaction.
 Δ, Sa-anti-Sa reaction.
 From (12).

As in the case of DEa, the reaction between DSa and anti-DSa was characterized by a sharp equivalence zone, typical of a homologous precipitin reaction between a single antigen and its antibody. The quantitative precipitin curve of the reaction was also characteristic of a single immunological entity.

In the region of antibody excess the ratio of DSa precipitated to DSa added ranged from about 80 to 90 per cent; which can be considered constant within experimental error. After removal of all antibody reactive with Sa, 82 per cent of the DSa added to the remaining antibody was precipitated, indicating that even if un-

changed Sa were assumed to be present in the dye, the amount could not exceed 20 per cent. Actually, it must have been much less since part, at least, of the non-precipitable N in DSa was derived from highly colored non-protein azo compounds.

From classical dye hapten studies it would be expected that DSa and DEa would cross react reciprocally. However, DSa failed to precipitate at any dilution with an antiserum to DEa and also failed to inhibit precipitation in the latter serum by the egg albumin dye. R-salt did not inhibit precipitation of DSa with anti-DSa and DEa did not precipitate anti-DSa.

It is apparent that while the introduction of a number of aromatic azo groupings into one protein may cause a profound change in specificity, introduction of the same groups into another protein may bring about so slight an alteration in specificity as to be undetectable in antiserum to the native protein.

While this difference in behavior between Ea and Sa was originally believed to signify that the tyrosine and other residues participating in the azo linkage are important to the specificity of Ea but not to that of Sa (12), another, and perhaps more likely, explanation has been advanced by Kendall (13), who pointed out that when Ea is spread in a film it is denatured and will not go back into solution, whereas serum protein when spread in films can be again obtained in solution. Thus, the forces holding Ea in globular form are weak so that once the molecule is distorted it no longer tends to reassume its original form. The forces in Sa are, however, strong enough to reestablish the globular form. If in the formation of the dye, Ea is opened up and takes an extended form, its specificity would be expected to change. With the Sa molecule, on the other hand, it is conceivable that even though the molecule might open up on coupling, the internal forces would be strong enough to make the azo compound reassume the globular form. The finding by Erickson and Neurath (14) that the specificity of Sa was not changed appreciably by treatment with urea also contrasts with the behavior of Ea on denaturation (see below) and may be interpreted as supporting Kendall's hypothesis (13).

Azo dyes containing hemolytic streptococcus protein (15): In the course of quantitative precipitin tests with protein fractions from group A hemolytic streptococci, it was observed that less nitrogen was precipitated than had been added as antigen N, and that

very large amounts of antigen were required to precipitate the maximal amount of antibody. To investigate the nature of this effect streptococcus antigen was labelled by coupling with the diazonium compound of R-salt-azo-biphenyl as had been done with crystalline egg albumin and serum albumin. Study of the chemical and serological properties of the red azo antigen showed that only a portion of the azo-protein reacted serologically, suggesting that a high proportion of serologically inert material of protein nature was present in the actual streptococcus protein antigen. Thus one could account at least in part, for the large quantities of streptococcus protein necessary for precipitation of antibody.

In contrast with antisera to DSa and DEa which contained no anti-hapten, immune serum against streptococcus azo-protein precipitated with both DSa and DEa, indicating the presence of antibody to the R-salt-azo-biphenyl hapten.

Phosphorylated egg albumin (PEa): The introduction of phosphoric acid groups into the Ea molecule by treatment with POCl_3 in the presence of alkali resulted in a marked change of immunological specificity without loss of antigenicity (17). PEa reacted strongly with anti-PEa. The reciprocal cross reactions, Ea-anti-PEa and PEa-anti-Ea, were slight and involved not more than 3 to 10 per cent of the total antibody in the sera tested. A further indication of the marked change in specificity as a result of phosphorylation was furnished by the failure of even very large amounts of Ea to inhibit precipitation of PEa with anti-PEa.

Tests of PEa with antisera to acid-denatured Ea (DnEa) and of anti-PEa with DnEa (see below) revealed extensive cross reaction, although PEa was not immunologically identical with DnEa. It is therefore evident that the changes noted in serological specificity were, at least partly, due to changes resembling those of acid denaturation as well as phosphorylation.

Phosphorylated serum albumin (PSa): A similar study on phosphorylation (18) was carried out with crystalline horse serum albumin. Difficulties were, however, encountered due to the lability of the phosphoryl groups in PSa. As in the previous study of PEa, it was found that PSa lost many of its bound phosphoryl groups in 2 to 3 days at 37°C . Even in the cold there was spontaneous loss of phosphoryl groups, although at a much slower rate. It was probable that hydrolysis also occurred during immunization of rabbits with

PSa so that antisera to PSa would be expected to contain antibodies at least in part to dephosphorylated PSa and not exclusively to the material actually injected. A direct study of the specificity of PSa using anti-PSa sera was therefore not possible.

Instead the cross reaction between PSa and anti-Sa was investigated. The results, plotted in fig. 25, indicated marked differences in specificity between Sa and PSa, as well as between different lots of PSa. Depending upon the extent of phosphorylation, different preparations of PSa precipitated from 20 to 90 per cent of the total anti-Sa.

The immunological changes produced in Sa by phosphorylation were found not to be fully reversible on dephosphorylation. If the phosphorylation of Sa involved only the attachment of phosphoryl groups, the immunological properties of partially dephosphorylated PSa should approach those of Sa in proportion to the loss in phosphorus. An increase of cross reactivity in anti-Sa was actually observed after partial dephosphorylation, but its magnitude was not commensurate with the loss in P, since the cross reaction of different products dephosphorylated at 37° C. fell below that of products of equal initial P content (Cf fig. 25). It would appear that some irreversible changes occurred on phosphorylation of Sa.

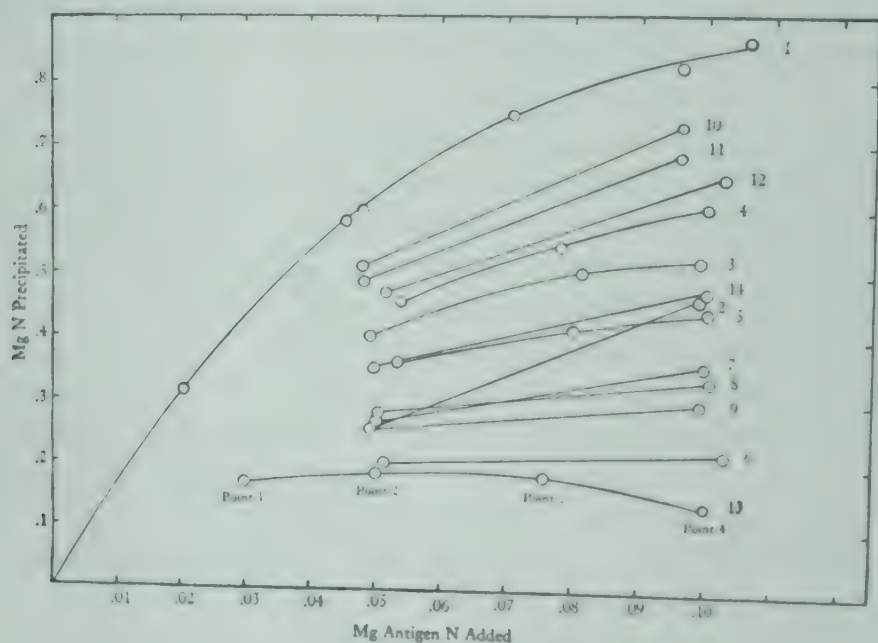


FIG. 25. Precipitin Reaction of Sa and PSa with 1.0 ml. of Rabbit anti-Sa Serum

CURVE NO.	PREPARATION NO.	N:P		REMARKS
		WEIGHT RATIO		
1	Sa			
2	PSa4B	9.0		Prepared with NaOH
3	PSa4C ₁	13.5	}	Derived from PSa4B by spontaneous dephosphorylation in the cold at pH 5.5. Separated by isoelectric precipitation into insoluble (C ₁) and soluble (C ₂) fractions.
4	PSa4C ₂	13.5		
5	PSa4D	9.1		Same as PSa4B but stored 4 mos. in the cold at pH 9 without spontaneous dephosphorylation.
6	PSa5B	7.2		Prepared like PSa4B but with more POCl ₃ .
7	PSa5E ₁	15.3	}	Derived from PSa5B by spontaneous dephosphorylation at 37°C and pH 7.4. Separated into fractions of low (E ₁), intermediate (E ₂) and high (E ₃) solubility.
8	PSa5E ₂	15.7		
9	PSa5E ₃	16.2		
10	PSa7	20.6		Prepared with sodium tetraborate.
11	PSa10S	14.7	}	Prepared with potassium tetraborate. Separated by isoelectric precipitation into soluble (S) and insoluble (P) fractions.
12	PSa10P	13.3		
13	PSa13	5.9		Prepared with NaOH. Most heavily phosphorylated lot.
14	PSa13A	24.7		Derived from PSa13 by spontaneous dephosphorylation at 37°C and pH 5.5.

Modified from (18).

It was also found that the relative fluidity (reciprocal of relative viscosity) of Sa decreased on phosphorylation, and that the decrease in fluidity ran parallel to the change in immunological specificity.

No evidence was found that antisera to PSa or PEa contained anti-hapten since there was no definite cross precipitation between the two phosphoproteins and since phosphate ion did not inhibit their precipitation by homologous antisera.

There are certain similarities between the response of Ea and Sa to phosphorylation and to coupling with R-salt. In each instance, Ea suffered a much more pronounced alteration in immunological specificity than did Sa. It is evident that the effects of chemical substitution on a protein depend on the properties of the protein as well as on the nature of the substituent. The hypothesis advanced by Kendall (13) in connection with the azo-protein studies may also be applicable to the phosphorylated proteins.

Studies on protein derivatives, therefore, in most instances yield only limited information on the relation between chemical structure

and serological specificity. This is for the most part due to the complexities of protein structure and it is evident that in many instances compounds of simpler composition, like polysaccharides, offer more immediate promise for investigations relating structure to serological specificity.

Serological properties of egg albumin denatured by acid, alkali, water and heat (19): These studies were carried out with preparations of acid-, alkali-, heat- and water-DnEa which had been prepared and purified under careful control (20), and had been characterized in the ultracentrifuge and by diffusion and viscosity measurements (21). It was found that the various kinds of DnEa

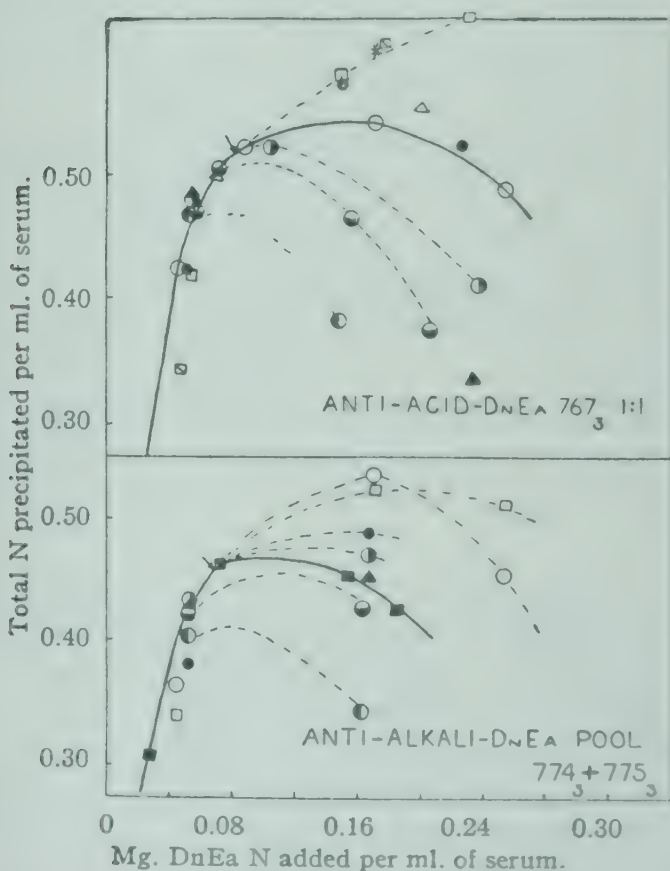


FIG. 26. Key to charts: ○, acid-DnEa 16; *, acid-DnEa 16 [1.5% NaCl]; ●, acid-DnEa 17; Δ, acid-DnEa 21; □, heat-DnEa 29; ▤, heat-DnEa 25; ▲, H₂O-DnEa; ⊙, alkali-DnEa 30; ⊖, alkali-DnEa 24; ⊕, alkali-DnEa 18; ■, alkali DnEa 23. Total N precipitated from 1.0 ml. of anti-DnEa rabbit sera by increasing amounts of various preparations of DnEa. Solid lines represent N precipitated by the antigens used for immunization: i.e., acid-DnEa 16 in the case of anti-acid DnEa 767-3 and alkali-DnEa 23 in the case of the anti-alkali-DnEa pool. Arrows show the points of approximate equivalence of homologous reaction. Ordinates do not start from origin of curves at 0,0. From (19).

were susceptible to extensive aggregation by exposure to salt and also that the state of aggregation varied with the age of the preparation.

The immunological characteristics of all kinds of DnEa regardless of the state of aggregation were identical when compared by quantitative precipitin tests in the region of antibody excess. This is evident from fig. 26 which summarizes data on the reactions of antisera to acid- and alkali-DnEa with various lots of acid-, alkali-, heat- and H₂O-DnEa. Differences were found, however, in the region of antigen excess. Thus, alkali-DnEa yielded less specific precipitate N with an antiserum to acid-DnEa than did the homologous antigen, although the amount of antibody N carried down was practically the same. Heat-DnEa, on the other hand, gave more specific precipitate N from the antiserum to acid-DnEa in the zone of antigen excess than did acid-DnEa. These differences permitted a serological distinction to be made between acid-, alkali- and heat-DnEa. In the region of antigen excess, H₂O-DnEa resembled alkali-DnEa in its reaction with antisera to acid-DnEa.

As in the case of anti-acid-DnEa, all varieties of DnEa were found to be equivalent in the region of antibody excess with antisera to alkali-DnEa. Differences were observed, however, in the zone of antigen excess.

The differences found in the antigen excess zone may be related, at least in part, to the state of aggregation of the antigen. Thus, lots of acid-DnEa which had been aggregated to a greater extent than usual by exposure to 1.5 per cent NaCl, gave more specific precipitate N than did the less highly aggregated lot of acid-DnEa which had been used to produce the antiserum. The larger amounts of N precipitated by the aggregated antigens appeared due to the larger masses of aggregate attached to the DnEa molecules in direct combination with the antibody. Tests on supernatants actually showed that less antigen remained in the supernatants from reactions with highly aggregated lots of acid-DnEa. The differences observed in the region of antigen excess are therefore probably not due to differences in serological specificity.

The value of quantitative analysis in the region of antigen excess was of limited significance because it was found that the longer the reaction mixtures were allowed to stand the more N was precipitated. This was not the case in analyses in the region of antibody

excess since reactions reached completion within 48 hours at 0-5° C., as usually occurs with other systems. It is thus apparent that the properties of DnEa impose limitations on the applicability of quantitative precipitin analyses.

Comparisons of different types of DnEa were also made in anti-Ea sera. Quantities much greater than those of the homologous Ea were required to produce reactions of the same order of magnitude. The extent of aggregation and degradation also appeared to influence the amount of precipitation. The more highly aggregated preparations gave more total precipitate N than did otherwise comparable but less extensively aggregated lots. With a lot of alkali-DnEa which had been subjected to severe degradation (1.2 per cent loss of N), reactivity with anti-Ea was almost completely abolished.

Cross reactions of Ea with antisera to DnEa (22) were weak and differed markedly from the homologous Ea-anti-Ea and DnEa—anti-DnEa reactions in their quantitative aspects. Much more Ea than DnEa was required for appreciable reaction.

On the whole, the various types of DnEa showed a strikingly uniform serological behavior, and in the region of antibody excess, in which all antigen added was precipitated, the various products appeared identical. Thus DnEa of all types would appear to have a definite and typical structural configuration, different from that of Ea, rather than a disordered state, (cf. 23).

Differences observed in the zone of antigen excess, however, appear ascribable largely to variations in the extent of aggregation and degradation of the preparations.

Immunochemical properties of native and denatured serum globulins from normal and immunized horses (24): Highly purified horse antibody to pneumococcus type I, obtained by salt dissociation and containing 73 to 85 per cent specifically precipitable N, and showing two components in the ultracentrifuge, was treated with 8 M guanidine hydrochloride for 24 to 36 hours at room temperature. After dialysis against 0.9 per cent NaCl solution, 85 to 90 per cent of the total protein was insoluble in all neutral solvents tested except in a solution containing 2 per cent NaCNS and 0.9 per cent NaCl. This insoluble fraction was considered as irreversibly denatured. The protein which remained in the supernatant after removal of the guanidine hydrochloride by

dialysis was believed to have been reversibly denatured and was accordingly termed the "regenerated" fraction.

Studies were also carried out with normal horse serum globulin GI (25) a fraction precipitated by ammonium sulfate at pH 6.4 within the concentration range of 1.1 to 1.36 *M*. Treatment with 8 *M* guanidine hydrochloride also converted 80 to 90 per cent of the protein to the "irreversibly" denatured form.

Antisera were prepared in rabbits using native normal horse serum globulin GI (NG), normal GI irreversibly denatured by 8 *M* guanidine hydrochloride (DG), native horse antibody (NA) and irreversibly denatured antibody (DA). DG was dissolved in a slight excess of alkali and then dialyzed against Ringer phosphate solution, pH 7.5, whereas DA was used as a suspension in saline both for immunization and for quantitative antibody determinations following the technique of Treffers and Heidelberger (26) (cf. also I,3).

It was found that DG reacted with about two-thirds of the antibody to NG, leaving antibody reactive only with NG in the supernatant. The constant *c*, in the equation

$$\text{Ab N pptd.} = cx - dx^{3/2}$$

(equation [5] in I, 2), was 14.4 for the homologous reaction with NG and 8.5 for the cross reaction with DG.

With anti-DG serum, the cross reacting antigen NG precipitated somewhat more antibody than did the homologous antigen, DG, and the values of *c* for the reactions with NG and DG were 10.0 and 8.5, respectively. This observation is most unusual and is not readily explainable, unless it is assumed, as suggested by Erickson and Neurath (24), that the antigenic properties of DG changed during the period between immunization and performance of precipitin analyses, or that the preparation of NG used for immunization contained some DG (24). It might also be possible that DG was contaminated with NG.

Erickson and Neurath (24) also studied the reactions of SI with "regenerated" and with "irreversibly" denatured antibody. Since solutions of the latter contained NaCNS, control experiments were also performed with native antibody in the presence of NaCNS to evaluate the effect of this substance on the course of the precipitin reaction. The results, which are plotted in fig. 27, indicated that the

course of the precipitin reaction between "regenerated" antibody and S I could be described by the equation (I,2):

$$\text{Ab N pptd.} = 2RS - \frac{R^2 S^2}{A}.$$

The characteristic constant, R , which describes the combining properties of antibody, was greater for the "regenerated" than for the native antibody, the respective values calculated from the equation being 7.8 and 4.6. This means that the "regenerated" antibody was less active than the native in that, on an equal weight basis, it combined with less antigen.

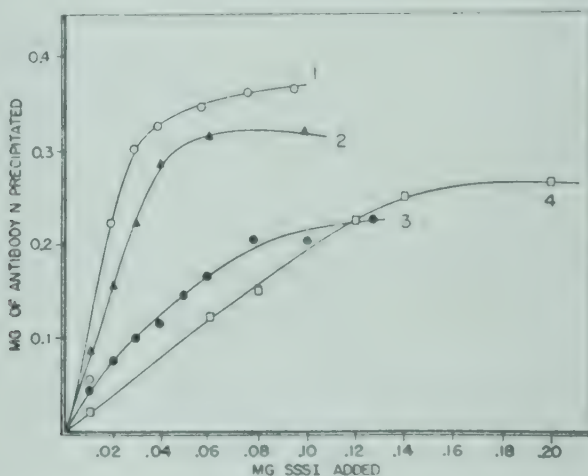


FIG. 27. Quantitative absorption of native, irreversibly denatured, and regenerated type I antipneumococcal horse antibodies by S I. All curves are reduced from the experimental data to the same content of total protein N (0.44 mg.). Curve 1 refers to regenerated antibody in 0.9 per cent NaCl, curve 2 to native antibody in 0.9 per cent NaCl, curve 3 to native antibody in 2 per cent NaCNS + 0.9 per cent NaCl, and curve 4 to irreversibly denatured antibody in 2 per cent NaCNS + 0.9 per cent NaCl. From (24).

This finding may be interpreted to indicate that the "regenerated" antibody had lost some of its reactive groups, possibly as a result of the aggregation in 8 M guanidine hydrochloride which was indicated by diffusion measurements (24). An alternative explanation may be offered, however, in view of the finding (24) that 83 per cent of the total protein in the "regenerated" antibody was precipitable by S I, compared to a purity of only 72 per cent for the native antibody from which it was made. Unless one chooses to assume that antibody has been synthesized by the treatment with guanidine hydrochloride, one would ascribe this increase to association of non-specific protein with antibody as a result of aggrega-

tion. Analogous observations are those of Weil, Moos and Clapp (27) who found that acidification of rabbit antipneumococcal sera to pH 4.0 or below, resulted in precipitation of an increased amount of nitrogen by specific polysaccharide, and of Wright (16) who found that diphtheria antitoxin after denaturation with urea yielded more precipitate with toxin although its neutralizing capacity for toxin was diminished (cf. also (31)).

The specificity of antigens containing two different, artificially introduced, determinant groups: A quantitative study was made by Haurowitz and Schwerin (28) using antisera prepared against the complex antigens arsanil-metanil-azo-sheep serum globulin (As-met-glob) and iodized arsanil-azo-globulin (I-As-glob), as well as to a mixture of arsanil-azo-globulin (As-glob) and iodo-globulin (I-glob). The simple arsanil and iodo derivatives of globulin and ovalbumin, as well as the complex As-I-ovalbumin were used as test antigens. The different antisera were analyzed by the quantitative precipitin technique for antibody to the various test antigens. The nature and the sequence of addition of the latter were chosen so as to yield information on the presence or absence of antibody to the various determinant groups as well as their amounts. A typical series of determinations is illustrated in table 2. To 3.0 ml. of immune serum against I-As-globulin the test antigens were added in portions of 1.2 mg. in sequence, as listed in table 2. After separation of the first precipitate by centrifugation, the supernatant was decanted as completely as possible, the precipitate was washed with 0.5 ml. of saline and the washing was added to the supernatant which was then mixed with the second test antigen, etc. Each of the precipitates was then washed three times with 2 to 3 ml. of 0.9 per cent saline and once with a mixture of methanol and water (1:1), with pure methanol, then with acetone, and finally with ether. After drying, the precipitates were weighed. Content of azo-antigen was determined colorimetrically after dissolving the weighed precipitate in 0.25 *N* NaOH with gentle heating. The iodo-antigen content of specific precipitates was determined by analysis for iodine.

It is seen from the results in table 2 that after stepwise removal of about 2.5 mg. of antibody protein reactive with the arsanil determinant group and about 2.3 mg. reactive with the iodo-tyrosine radical, subsequent addition of I-As-ovalbumin caused no additional

precipitation, indicating that there was no antibody fraction reactive exclusively with di-substituted test antigen, i.e., not with antigen containing only one or the other determinant group. Subsequent reaction with globulin revealed the presence in the antiserum of about 7.1 mg. of antibody specific for the carrier protein. Finally,

TABLE 2

Fractional Precipitation of 3.0 ml. of Immune serum to I-As-globulin

Test antigen	Antibody precipitated	Antigen precipitated
Experiment No. 1		
	mg.	mg.
As-ovalbumin.....	2.017	0.31
As-ovalbumin.....	0.516	0.12
I-ovalbumin.....	2.281	0.28
I-ovalbumin.....	trace	
I-As-ovalbumin.....	0	
Globulin.....	5.481	
Globulin.....	1.665	
Globulin.....	trace	
I-As-globulin.....	2.264	0.50
I-As-globulin.....	0	
Total.....	14.22	
Experiment No. 2		
I-As-ovalbumin.....	5.360	0.58
I-As-ovalbumin.....	trace	
Globulin.....	6.383	
Globulin.....	1.127	
I-As-globulin.....	2.472	0.45
I-As-globulin.....	trace	
Total.....	15.34	

Modified from (28)

The test antigens were added in portions of 1.2 mg. in the sequence listed.

another 2.7 mg. of antibody was recovered with the homologous antigen, I-As-globulin, indicating the presence of an antibody fraction directed in specificity toward the substituent groups plus some part of the globulin molecule.

In a second experiment (table 2), I-As-ovalbumin was added first and precipitated about 5.4 mg. of antibody, as compared with 4.8 mg. ($2.5 + 2.3$) obtained in the first experiment by successive analyses with As-ovalbumin and I-ovalbumin. Subsequent addition of globulin in the second experiment yielded about 7.5 mg. of antibody, as compared to 7.1 mg. in the first experiment. Finally, the homologous antigen reacted with 2.5 mg. of antibody which is close to the value 2.7 obtained in experiment No. 1. It would seem likely that even better quantitative correspondence would have been obtained if the amounts of antigen added at each step had been reduced progressively to avoid the possibility of inhibition. It is also possible that univalent antibody was in part responsible for the differences.

It may be concluded, therefore, that immune sera contain a mixture of antibody fractions of different specificities reflecting the various determinant groups of the antigen. The extent to which resolution of an immune serum into its constituent antibody fractions may be accomplished appears to be a question largely of the availability of specific test antigens. When cross reactions take place between naturally occurring single antigens, they may also be ascribed to the presence of an antibody fraction reactive either with an identical antigenic determinant grouping shared by the cross reacting antigens, or to a structurally similar molecular grouping present in both antigens (Cf II, 9—Fractionation of antibody to pneumococcus S VIII by means of the cross reacting polysaccharide, S III).

The effect of partial hydrolysis on the serological properties of pneumococcus S III (29): Products of partial hydrolysis of S III ranging from 550 to 1800 in formula weight were separated by fractional alcoholic precipitation and quantitatively freed from unhydrolyzed polysaccharide. It was found (29) that these fractions gave precipitates with horse anti-S III but failed to precipitate rabbit anti-S III. They did, however, in high concentration, inhibit precipitation of S III with rabbit antibody. The aldobionic

acid, glucurono-glucose, or cellobiuronic acid, the structural unit of SIII, did not precipitate with antisera.

In horse sera only a portion of the anti-S III was precipitated by the cleavage products, the extent of cross reaction varying inversely with the degree of hydrolysis, indicating that antiserum to S III contains antibody fractions of a specificity directed toward groupings characteristic of intact S III. Hence, the serological specificity of an antigen cannot be accounted for solely in terms of its basic constituents but the over-all structure of the antigen is also of importance. Moreover, it is evident that antibody formed in different species may possess radically different properties (Cf. IV, 51).

Methylated pneumococcus S III (30): S III was methylated with methyl sulfate and NaOH. The product was found to give precipitates with horse-anti-S III but did not precipitate with the corresponding rabbit serum. The reaction of rabbit antibody with S III was not inhibited by the methylated polysaccharide. Data from (30), given below, show that the methylated S III precipitated only about two-thirds of the horse antibody to S III.

Nitrogen precipitated by methylated S III from a horse antibody solution to pneumococcus type III (Total anti-S III = 1.25 mg. N/ml.) (30)

Methylated S III	N pptd.	Supernatant tested with:		
		antibody	methyl-S III	S III
mg.	mg.			
0.05	0.43	—	++	
0.10	0.60	+	+	
0.15	0.68	±	±	
0.25	0.74	++	±	++
0.50	0.83	++	—	++

It may be noted from the supernatant tests that the antibody not thrown down by methyl-S III could be precipitated by native S III. This residual antibody still failed to react with methyl-S III after concentration by Felton's method (IV, 43), but it did precipitate with fractions of partially hydrolyzed S III. These findings were among the first to indicate that antibodies formed against a single antigen vary in the nature and number of their reactive groups (Cf. I, 2, 6).

Effect of formaldehyde on antigens and antibodies: The

interaction of formaldehyde with proteins is as yet little understood, but it appears to involve as an initial step the formation of methylene linkages of the type $\text{CH}_2 = \text{N}-$ with aliphatic amino groups (32). It can be shown by amino group determinations according to Van Slyke (III, 14) or by formol titrations (III, 31) (33) that amino groups (chiefly the $\delta\text{-NH}_2$ of lysine) are blocked; other groups, however, such as indole nuclei and imidazole rings may also react (34, 35). The reaction is dependent on pH, being most intense in alkaline media, and can be reversed, at least in part, in acid solution.

It is well known that treatment of diphtheria toxin and many other toxins with formalin destroys the toxic properties without appreciably affecting the capacity to flocculate with antitoxin, or to stimulate the production of antitoxin on injection. The behavior of pure diphtheria toxin (IV, 49) has been investigated by Pappenheimer (37) who found, in agreement with Eaton (38), that a low concentration of formalin (0.2 per cent) at pH 8.1 destroyed the toxicity in 6 days at 38°C . but did not abolish ability to flocculate with antitoxin, although the speed of flocculation was decreased. Toxicity was also abolished by 1 per cent formalin at pH 6.3. The reduction in free amino nitrogen of the diphtheria toxin on treatment with formaldehyde corresponded to the lysine content. The effect of formaldehyde was found to be irreversible so that it cannot be attributed merely to the formation of methylene linkages.

Formation of toxoid does not parallel the reaction between amino groups and formaldehyde, as measured by the Sørensen titration, in that the former reaction is irreversible and requires amounts of formaldehyde equal to or less than those theoretically necessary to combine with all amino groups, while in the Sørensen titration a large excess of formaldehyde must be used to obtain rapid and complete interaction.

The effect of formaldehyde on the specificity of serum proteins was studied by Landsteiner and Jablon (39), and Landsteiner and Lampl (40), who observed no pronounced change in specificity such as is produced by halogenation, nitration or coupling with diazo compounds. Formolinized serum still precipitated with antisera to native serum, and antisera to the formolinized antigen reacted with the native protein. A change was noted, however, in that injection of formolinized rabbit serum into rabbits produced

antibodies which reacted with the modified antigen. Horsfall (41) confirmed these observations and in addition found that antibodies against formolinized serum precipitated with formolinized sera from serologically unrelated species, although less intensely than with homologous native or formolinized serum. Similar results were obtained by anaphylactic tests with uterine strips from passively sensitized guinea pigs. It would appear therefore that formolinized proteins possess a new, characteristic specificity, although they have not lost their species specificity. A number of formolinized amino acids failed to inhibit precipitation (41). In a more recent investigation Jacobs and Sommers (42) confirmed Landsteiner's findings (39, 40), and pointed out that Horsfall's observations of serological relationships among formolinized proteins from various species may have resulted from protracted treatment involving more extensive changes than those produced by Landsteiner (39, 40).

Viruses generally lose their infectivity on treatment with formalin. However, Stanley (43), and Ross and Stanley (35) found that tobacco mosaic virus inactivated by formaldehyde can be crystallized and still retains its ability to precipitate with antisera to native virus (43). Destruction of infectivity was accompanied by a decrease in amino groups as indicated by Van Slyke nitrous acid analyses (III, 14), and by a diminution in reactivity with the Folin reagent (III, 22) at pH 7.7 (35). The latter finding was interpreted to indicate reaction of formaldehyde with indole nuclei of tryptophane.

Partial reactivation accompanied by an increase of amino nitrogen and of groups reactive with the Folin reagent, could be accomplished by dialysis at pH 3 (35).

Small concentrations of formaldehyde have been found to destroy the flocculating ability of diphtheria antitoxin (44) and of precipitins to pneumococcal type-specific polysaccharides (45, 46). Formolinized antipneumococcus serum also lost its capacity to agglutinate pneumococci. The formolinized antibodies, although unable to flocculate, precipitate or agglutinate, could still enter into specific combination with antigen. Chow and Goebel (45) also showed that the effect of formaldehyde could be only partially reversed by exposure of formolinized antibody to pH 4 at 0° C. for

several days, another indication that changes other than mere formation of methylene linkages may have occurred. Chow and Goebel's findings (45) were confirmed by Heidelberger, Grabar and Treffers (47) who again emphasized the importance of control of pH in the treatment of proteins with formaldehyde (cf. 46).

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CHAPTER II

COMBINED USES OF SEVERAL METHODS

Quantitative agglutinin and precipitin techniques, when employed in conjunction with tests for biological activity, such as protection, neutralization or toxicity assays, are of great value in the study of complex mixtures of antigens like bacteria, since they make it possible to account for and follow the various constituents obtained by chemical fractionation and establish their relationship to one another. Data obtained at each step in the course of preparation may indicate whether all of the original immunological activity is contained in the constituent fractions or whether loss of serological activity has resulted from chemical treatment. This type of investigation has furnished information about the presence of unknown antigens in microorganisms and in other instances, has indicated that certain substances had been isolated in the form in which they actually occurred in the intact organism. Several illustrations of the combined use of the procedures in studying the chemistry of bacteria will be given.

Identity of agglutinin and precipitin: (I) The type-specific anti-carbohydrate in type I horse antipneumococcal sera can be measured directly by the quantitative precipitin test using type-specific carbohydrate (I). It may also be estimated as the difference between the total agglutinin N using a suspension of I S (Dawson M) pneumococci and the group-specific agglutinin N with type I R organisms, which could be shown not to contain any type-specific polysaccharide (I, 3; II, 7).

After removal of the group-specific antibody with I R organisms type-specific anti-carbohydrate could be estimated in the supernatants, either by quantitative precipitin tests with polysaccharide, or by quantitative agglutinin estimations with suspensions of type I S pneumococci. Table I shows the results obtained with type I horse antipneumococcal sera and with Felton antibody solutions (cf. I, 6). The table shows that the type-specific precipitin plus any residual group-specific antibody in the supernatant equals total agglutinin and that the same total amount of antibody nitrogen is also obtained when a portion of the type-specific antibody is removed as precipitin or agglutinin and the remainder as agglutinin

or precipitin. Within the limits of accuracy of the methods, the data indicate the quantitative correspondence or identity of the type-specific anticarbohydrate agglutinin and precipitin in the horse sera. Since several sets of analyses are involved in obtaining the values for total antibody N content by combined agglutinin and precipitin estimations the precision is not as great as in the estimation of total agglutinin N or total precipitin N. The identity of agglutinin and precipitin in type I-III dysentery antiserum has recently been established by Perlman and Goebel (1a).

The finding in horse antipneumococcal sera that agglutinin and precipitin were identical suggested that with horse antisera the precipitating power of the purified polysaccharide preparation used for the precipitin tests was the same as that in the suspension of organisms used for agglutination. Hence, it might have been inferred that the polysaccharides had actually been obtained in an undegraded form. This would have been incorrect, since the same polysaccharides were found to vary widely in their capacity to precipitate rabbit antipneumococcal sera (2). With rabbit antisera the identity of agglutinin and precipitin could not be demonstrated until the effects of heat, acid and alkali on the immunological activity had been disclosed and polysaccharides had been prepared using mild procedures (2) (IV, 51). Thus Chow (4) found that type I polysaccharide prepared with trichloroacetic acid could precipitate only 35 per cent of the total agglutinin N in a type I antipneumococcal rabbit serum. With polysaccharides obtained by mild methods, however, the identity of the anticarbohydrate precipitin and agglutinin in rabbit antisera could be established (3, 5).

The data for the Felton antibody solutions in Table I show that after complete removal of the precipitin N with type-specific carbohydrate, a small but definite amount of agglutinin N always remained in the supernatant. This has also been found for antibody solutions purified by various methods (6, 7), and has been taken to indicate that a small portion of the antibody is altered in the course of purification so that it no longer precipitates with carbohydrate but is carried down specifically on a suspension of bacteria (cf. I, 6). The quantitative methods, therefore, are sufficiently sensitive to disclose slight differences in the properties of antibodies as a result of purification.

TABLE 1
Quantitative Comparison of Agglutinin and Precipitin in Type I Antipneumococcus Sera and Antibody Solutions

Laboratory designation of serum	Volume of serum or antibody solution used	Agglutinin removed by Pn I	Strain used	Precipitin found in supernatant	Remaining antibody N with Pn I S suspension	Total antibody N	Antibody N per ml.	Volume of serum or antibody solution used	Precipitin removed	Agglutinin in supernatant	Strain used	Remaining antibody N removed by Pn I S suspension	Total antibody N	Antibody N per ml.
	ml.	mg.		mg.	mg.	mg.	mg.	ml.	mg.	mg.		mg.	mg.	mg.
Serum H 610	0.50	0.77*	I S	0.00	0.00	0.77	1.54	0.50	0.75†	0.05	I R	0.00	0.80	1.60
	0.50	0.32	I S	0.38	0.05	0.75	1.50	0.50	0.75†	0.02	I R	0.00	0.77	1.54
	0.50	0.25	I S	0.50	0.06	0.81	1.62	0.50	0.52††	0.26	I S	0.01	0.78	1.56
	0.50	0.16§	I R	0.59†	0.00	0.75	1.50	0.50	0.60	0.21	I S	0.00	0.82	1.64
Serum H 701 (1:1)**	1.00	1.16	I S	0.00	0.00	1.16	1.16	1.00	1.05	0.14	I S	0.00	1.19	1.19
	1.00	0.27	I S	0.73	0.16	1.16	1.16	1.00	1.05	0.13	I R	0.00	1.18	1.18
	1.00	0.33	I R	0.81†	0.03	1.17	1.17	1.00	0.89	0.31*	I S	0.00	1.20	1.20
	0.50	0.82*	I S	0.00	0.00	0.82	1.64	0.50	0.75	0.01	I R	0.00	0.76	1.52
Antibody B 76	0.50	0.53	I S	0.08	0.17	0.78	1.56	0.50	0.55	0.24	I S	0.00	0.79	1.58
	0.50	0.09§	I R	0.61†	0.07	0.77	1.54	0.50	0.52	0.10	I S	0.00	0.62	1.24
	0.50	0.61*	I S	0.00	0.00	0.61	1.22	0.50	0.54†	0.06	I S	0.00	0.60	1.20
	1.00	0.02	I R	0.32	0.12*	0.60	1.20	0.50	0.35	0.25	I S	0.01	0.61	1.22
Antibody B 76 absorbed	1.00	0.69	I S	0.00	0.07	0.69	0.69	1.00	0.69††	0.05	I S	0.00	0.74	0.74
	1.00	0.30	I S	0.33	0.07	0.70	0.70	1.00	0.63†	0.10	I S	0.00	0.73	0.73
									0.56	0.11	I S	0.02	0.69	0.69

* Two absorptions.

† Complete removal of anti-S as shown by excess S in supernatant.

†† 2 hours at 37° C. Ice box overnight.

§ A second absorption with Pneumococcus I R removed no more antibody.

From (1)

|| Three absorptions.

** Contained 0.13 mg. anti-CN per ml.

† Broth culture filtrate of Pneumococcus I S used.

With antibody concentrates or sera that have been subjected to chemical treatment the possibility that the precipitating properties of the antibody may have been altered must always be considered. This is sharply illustrated by the results of Weil, Moos, and Clapp (8) who found that acidification of rabbit antipneumococcal sera to pH 4.0 or below resulted in precipitation of an increased amount of nitrogen per ml. of serum by specific polysaccharide. This was accompanied by impairment or loss of complement-fixing power and by lower ratios of mouse protective units per milligram N precipitable by polysaccharide. These changes are probably attributable to addition complexes of the antibody and other serum proteins. Kleczkowski also showed that rabbit antibodies form complexes with other proteins on heating and that these complexes combined with antigen to varying degrees. Antibody complexes with globulin flocculated with antigen, while complexes with albumin did not flocculate but inhibition tests established that they were still capable of combining with antigen (9). Electrophoretic studies of heated sera showed the conversion of the various components to a single component with the mobility of beta globulin (10, 11) thus lending independent support to the immunological observations.

Demonstration of the presence of unknown antigens in bacteria: In the case of the type I meningococcus, an integrated study of the type-specific and group-specific and protective antibodies in antisera to the intact organisms sufficed to establish the existence of a type-specific antigen in addition to the Type I polysaccharide. This new antigen was responsible for the larger part of the protective power of rabbit and chicken antimeningococcal sera (12).

The total agglutinin content of Type I antimeningococcal sera was determined with a suspension of Type I organisms and the total group-specific antibody was measured with a Type II suspension. Protection tests on the supernatant sera served to demonstrate that all or almost all of the protective antibody was removed by the homologous organisms whereas little or no protective antibody was removed by bacteria of the heterologous type, although in some sera the group-specific antibodies constituted as much as half of the total antibody N. Quantitative precipitin determinations with Type I meningococcal polysaccharide established that only a small proportion of the total antibody N content of rabbit

and chicken antisera was antipolysaccharide, and protection tests on the supernatant after removal of the antipolysaccharide indicated that only a small proportion of the protective power had been removed. Data are given in table 2. It is apparent that these antisera contain relatively large amounts of protective antibody other than that reactive with the Type I polysaccharide preparations used, and the existence in the organism of a type-specific antigen other than the polysaccharide may be inferred. This antigen has not yet been isolated.

With Type I horse antimeningococcal sera, the antipolysaccharide comprised a much larger proportion both of the total antibody and of the protective antibody. Scherp and Rake (13) found that with some horse antisera, the antipolysaccharide could account for 87 to 99 per cent of the protective power.

Sera of all species studied showed a relatively high proportion of group specific antibody. This was especially true for the sera of patients convalescing from meningococcal meningitis in which about the same amounts of Type I and Type II agglutinin were generally found (12).

From table 2 it is apparent that removal of the Type I anti-

TABLE 2

Antibody Nitrogen and Mouse-Protective Power Removed from Antimeningococcus Horse (H), Rabbit (R), and Chicken (C) Sera by Types I and II Meningococci and by Purified Type I Meningococcus Polysaccharide

Serum	Antiserum to meningococcus type	Antibody N removed per ml. of serum by				Percentage of protective power against Type I meningococci in supernatant after absorption with			
		Meningococcus cells		Type I polysaccharide		Meningococcus cells		Type I polysaccharide	
		Type I	Type II	Preparation 18 (Scherp)	M6C or M9B ₂	Type I	Type II	Preparation 18 (Scherp)	M6C or M9B ₂
		mg./ml.	mg./ml.	mg./ml.	mg./ml.	per cent	per cent	per cent	per cent
H1095.....	I	1.15	0.70	0.71	0.34	0	100	60-70	40-50
H antitoxin..	?	0.56	0.58						
Rx (pool)....	I	0.24	0.11	0.04	0.015	0	100	90-100	90-100
RL.....	I	0.50	0.01	0.11	0.09	10	100	50†	50-100
C84 ₁	I	0.49	0.25		0.03*				
C84 ₂	I	0.25	0.16	0.002*	0.002*				
C85 ₁	I	0.55	0.28	0.061*	0.049*	0	90	90-100	90-100
C135.....	II	0.20	0.32		0.005*				

* Determined by micro method using Folin-Ciocalteu reagent.

† A 1/1600 dilution of this supernatant protected against 100,000 lethal doses, as compared with a 1/3200 dilution of a control of serum with saline.

From (12)

polysaccharide and the group-specific agglutinin from any of the sera would provide a specific reagent for the detection of the new type-specific protective antigen. Such an absorbed serum should

prove valuable for detecting this antigen during chemical fractionation, as well as for determining whether any purified preparation still retained its native immunological properties by testing its capacity to remove all of the protective power from the serum. This latter criterion was used by Zittle (14) who demonstrated that after removal of the antibody from antisera to whole streptococci with homologous purified M protein, the supernate failed to protect mice against 1 MLD although an equivalent quantity of the whole serum had protected against 10 MLD. By essentially similar procedures it was established that the protective power of rabbit antisera to *H. influenzae*, Type B, could be completely eliminated by removal of the antipolysaccharide (18) and that the protective antibodies in antisera to *Sh. paradysenteriae*, Type III were associated almost exclusively with antibody to the Type III specific complex antigen (19). The d-glutamyl polypeptid of anthrax bacillus, as well as two specific polysaccharides, were shown not to remove protective antibody from anti-anthrax sera (20).

Suggested procedure for use of immunochemical methods as a guide to the fractionation of bacteria or other microorganisms: The specific illustrations given above should indicate the utility of these methods in the study of complex mixtures of antigens such as bacteria. Since this approach has been applied as yet in only a few instances, it may be of value to outline in general terms how one might best proceed with the study of any microorganism.

1. Antisera should be prepared in a suitable species, generally rabbits, horses, chickens, to the intact organism under investigation. The suspensions used for immunization should be killed by mild methods to avoid changes in antigenicity (I, 3). Formalin, merthiolate, acid, and in some instances heat-killed suspensions have been used (I, 3), but the latter are not generally recommended. Possible effects of the method of killing the organisms on their antigenic behavior must always be considered. With non-pathogenic organisms, or after some immunity has developed to certain pathogens, suspensions of living bacteria may be employed.

If organisms are not grown on a synthetic medium, cultures should be washed free from antigens present in the culture medium. Frequently, these substances cannot be completely eliminated, since the bacteria may incorporate them into themselves. This has

been found to be the case for agar (15) serum (16) and tissue proteins (17). If possible, media containing such materials should be avoided. When this is not practical, broth cultures may be employed for quantitative agglutinin determinations with antisera prepared from cultures on agar or vice versa and cultures may be grown in media prepared with serum of one species for immunization and serum of another species for antibody estimations. Adequate consideration of such matters in planning the investigation should materially simplify the interpretation of results.

2. The total antibody content of the antisera should be assayed by the quantitative agglutinin method (I, 3) and their protective power determined, if possible. The ability of the intact organisms to remove all of the protective antibody should be established by protection tests on the supernatant serum from the quantitative agglutinin estimations.

3. If microorganisms of closely related species are available, quantitative agglutinin determinations and tests for protective power on the supernatant will yield information on the relationship between the cross reacting antigens and protective power as outlined above for R and S pneumococci or for Types I and II meningococci. Should these heterologous suspensions fail to remove protective antibody, absorption of the antisera with these suspensions will render them more specific and facilitate their use as guides in chemical fractionation of the antigens associated with protection. Conversely, should protective power be found to be associated with the cross reacting organisms, it might prove desirable to use antisera to the heterologous organisms in testing for the protective antigens, provided these antisera contain fewer of the homologous antigens which are not associated with protection.

4. At each step in the chemical fractionation of the microorganisms, precipitin tests with the various antisera will furnish valuable information. Serologically inert fractions may be detected by failure to obtain precipitin tests with antisera to the whole organism. Group-specific antigens may be demonstrated by tests with heterologous antisera; antigens specific for the homologous organism may be discovered by tests with absorbed antisera; and antigens associated with protective power may be recognized by decreases in protective power of supernatants from quantitative precipitin

determinations. In many instances, quantitative estimation may not be necessary.

5. As chemical fractionation proceeds and relatively well defined products are obtained, they may be used to expedite further studies. If a given product is not associated with protection, it may be used to absorb the serum, thus rendering it more specific with respect to the protective antigens. In this way it may become possible to obtain a serum specific for the protective antigen and to use such a serum to detect the protective antigen in various products by precipitin tests alone. Needless to say, however, the final test of any product, supposed to be the protective antigen, will be its capacity to remove all of the protective antibody from the antiserum.

6. When a product is obtained which is capable of removing all of the protective power from antisera, information about its purity, in addition to the usual chemical and physicochemical criteria may be obtained by a study of its quantitative precipitin curve with antisera to the intact homologous microorganism (cf. II, 8), by tests with heterologous antisera and with antisera to other purified fractions (II, 7).

7. Finally, its effectiveness as an antigen should be established, since its ability to induce antibody formation may have been reduced as a result of purification without alteration of its reactivity with antisera.

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Part III

CHEMICAL AND PHYSICAL METHODS AND SPECIAL PROCEDURES USED IN IMMUNOCHEMISTRY

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CHAPTER 12

KJELDAHL NITROGEN DETERMINATION

Protein is usually determined by indirect methods rather than by isolation and weighing, because complex procedures are necessary for separation from accompanying constituents such as salts, and because it is difficult to obtain proteins in a well defined state of dryness. (1).

Because nitrogen is an integral constituent of all proteins and since many proteins have very similar nitrogen contents, quantitative analysis for protein is conveniently carried out by estimation of nitrogen. In mixtures containing considerable amounts of nitrogenous matter other than protein, it is, however, necessary to determine the total nitrogen, separate the protein and then estimate the non-protein nitrogen. This may be done with the aid of protein precipitants, such as trichloroacetic acid or phosphotungstic acid or by coagulation of the protein by heat. Protein nitrogen is thus obtained as the difference between total nitrogen and non-protein nitrogen (2).

In certain instances other methods may be used for estimation of protein, such as the Folin-Ciocalteu phenol reagent (III, 22), density determination (III, 23), refractive index measurements, or ultraviolet absorption spectra (III, 30). They are generally standardized relative to nitrogen and the results are frequently expressed as nitrogen.

Nitrogen analysis by the Micro-Kjeldahl method: The substance is decomposed by boiling with concentrated H_2SO_4 , K_2SO_4 and a catalyst such as Cu, Se or Hg. Nitrogen is thus converted into $(\text{NH}_4)_2\text{SO}_4$. After addition of excess alkali, the NH_3 is steam-distilled into acid and then titrated. Classical technique calls for distillation into an excess of standard HCl and back titration with standard NaOH solution, but this necessitates two precise measurements. One of these may be eliminated by the use of boric acid (3) to receive the NH_3 and titration of the ammonium borate which is formed with standard HCl. The boric acid need not be measured precisely. Since boric acid is extremely weak, it is displaced from ammonium borate by HCl. Excess of HCl turns the

methyl red indicator red. Boric acid is so weak it barely affects the indicator, but ammonium borate turns the indicator yellow.

Limitations: In substances containing nitro-, nitroso-, or azo-groups, and in hydrazines, N cannot be determined quantitatively unless these groups are first reduced (5). Elek and Sobotka (4) employ glucose (100 mg. per determination) for this purpose, but it is inadequate for hydrazines. Any nitrogenous compound, including the hydrazines, can be determined by reduction with P and HI, followed by digestion with H_2SO_4 , mercuric acetate and K_2SO_4 (5). Nitrates, however, escape measurement because HNO_3 is volatile. Certain proteins, e.g. hemoglobin, do not yield all their nitrogen in Kjeldahl analysis using a copper catalyst. The use of a selenium catalyst (6) overcomes this difficulty.

REAGENTS

1. **Sulfuric Acid**, containing CuSO_4 catalyst. Add about 40 ml. saturated aqueous CuSO_4 solution to a 9 lb. bottle of "low N" C.P. reagent sulfuric acid in 10 ml. portions with thorough mixing. After several days, excess anhydrous CuSO_4 crystallizes out, is allowed to settle, and the supernatant acid is ready for use.
2. **Potassium Sulfate:** Analytical Reagent, low nitrogen.
3. **Boric acid-indicator mixture:** Two ml. of a saturated solution of twice recrystallized methyl red in 50 per cent alcohol is added to 100 ml. saturated boric acid solution. 5 ml. of solution are added to 5 ml. of water for each analysis.

Alternative indicator mixture giving better color change near end point:

Stock solution A — 1% methylene blue in water.

Stock solution B—Saturated solution of twice recrystallized methyl red in 95% ethyl alcohol.

Stock solution C—Mix 15 ml. of stock solution A with 125 ml. of solution B. These proportions generally give optimum sharpness of endpoint, but may require adjustment with different lots of dye.

Solution D—Add 5 to 10 ml. of solution C to 2 liters of saturated boric acid. Use 5 ml. "D" with 5 ml. water for each analysis.

4. **Saturated NaOH Solution.** Remove insoluble Na_2CO_3 by filtration through fluted paper. Keep in closed vessel.

5. **Standard N/70 HCl:** Prepared exactly by direct weighing of constant boiling HCl (7).

A simple procedure for preparing constant boiling HCl is given by Bonner and Branting (7). The composition, which depends solely on the barometric pressure on the day of preparation, may be ascertained from Table 1. For the preparation of 1 liter of exactly N/70 HCl, the amount indicated in column 4, Table 1, should be weighed exactly into a small ground-glass stoppered vial. Transfer quantitatively to a calibrated 1000 ml. volumetric flask and make up to volume.

TABLE 1

Preparation of Standard HCl from Constant Boiling HCl
(After Foulk and Hollingsworth⁶)

Barometer, mm.	Per cent HCl in acid	Grams of acid, weighed in air, required for 1 liter of 1.0 N acid	Grams of acid, weighed in air, required for 1 liter of N/70 acid
770	20.197	180.407	2.577
760	20.221	180.193	2.574
750	20.245	179.979	2.571
740	20.269	179.766	2.568
730	20.293	179.555	2.565

Notes. The methyl red for solution 3 should be recrystallized twice from ethyl alcohol before use. The boric acid-methyl red mixture keeps indefinitely in pyrex vessels (3).

Solution C will keep for months. Solution D changes with precipitation of methylene blue. It should therefore be made up fresh every few weeks and is best kept in pyrex glassware.

PROCEDURE

- A weighed or measured sample is placed in a Kjeldahl flask. Add:
- (1) 2 ml. of sulfuric acid solution saturated with copper sulfate.
 - (2) A few large crystals of potassium sulfate (approx. 1 gm.).
 - (3) One undamaged, spherical glass bead of 4-5 mm. diameter or a boiling stone.

Digestion is carried out on a rack like the one illustrated in figure 28. Place the flask on burner at a 45° angle and bring to a gentle continuous boil over a medium flame. In case of foaming, boil as gently as possible. If foaming persists, add several drops of octyl

alcohol or 2-ethylhexanol. Foaming may become especially serious when large volumes of sample are used as in the quantitative precipitin and agglutinin methods, and in some instances it may be advisable to evaporate off the water on a steam bath or in an oven. Do not permit boiling to stop at any time since superheating and "bumping" will result. After the water is boiled off, SO_3 fumes

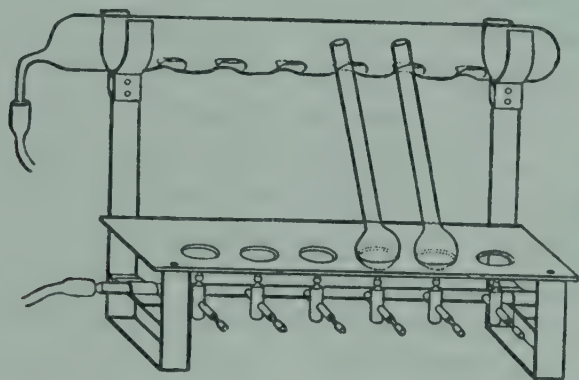


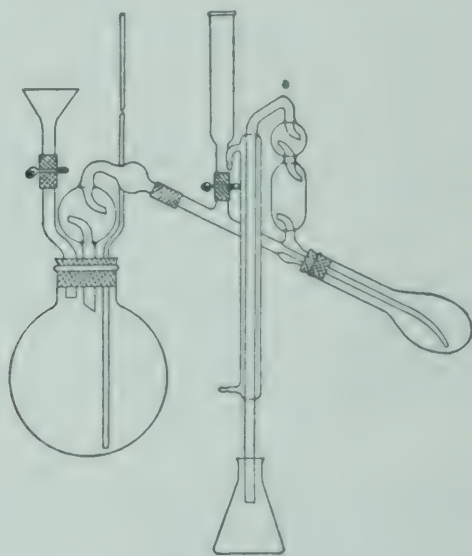
FIG. 28. Digestion rack for Micro-Kjeldahl determinations. *From (5).*

appear and charring frequently occurs. As digestion progresses the solution clears and becomes colorless. (At this time the neck of each flask should be inspected carefully for undigested black residues. If any undigested material remains in the neck, allow the flask to cool, wash down the neck with water and resume heating with constant shaking of the flask until boiling begins.) Boiling is continued for $\frac{1}{2}$ hour after all particles have disappeared and the solution has become clear and colorless. Allow to cool to room temperature, then cool in ice-water, and when cold, dilute contents of flask with approximately 10 ml. of water. Flasks should be watched continuously during the early stages of digestion, but may be left after SO_3 fumes appear.

Before use, the Kjeldahl apparatus (fig. 29) is steamed out for 5 minutes, with a Kjeldahl flask in place containing 10 ml. of distilled water and 9 ml. of alkali. If the distillate is neutral to methyl red, remove flame, and run a blank as follows:

Place a 125 ml. Erlenmeyer flask containing 5 ml. of boric acid-indicator mixture and 5 ml. of water under the condenser, with the tip of the latter barely touching the surface of the liquid. Place a Kjeldahl flask containing 10 ml. of water on the apparatus. Run 9 ml. of alkali into the Kjeldahl flask, replace flame under boiler and immediately raise flask of boric acid until the condenser touches the

bottom tilting the flask so that the tip of the condenser is covered as deeply as possible. After 6 minutes of distillation, lower the Erlenmeyer flask, wash off the condenser-tip and remove the flame. After all water accumulated in the trap on the right (fig. 29) has returned to the Kjeldahl flask, replace flame and distill for one minute more, leaving the condenser tip above the level of the liquid in the flask. This blank run serves as a reference standard for indicator color. If its color is satisfactory (faintly red, or grey or slightly purple with the alternative indicator), place another 125 ml. Erlenmeyer flask with 5 ml. of boric acid-indicator mixture and 5 ml. of water under the condenser. Attach the Kjeldahl flask containing the analytical sample diluted with water and chilled in a pan of ice water, making sure of a leak-proof connection, and proceed as in the blank run. Distillations are best run in a continuous series. In case of doubt about the color of the blank, additional blanks are distilled in series until satisfactory results are obtained. Distillation of ammonia turns the boric acid indicator solution yellow (or green with alternate indicator solution).



—Courtesy of Eck and Krebs, New York

FIG. 29. Micro-Kjeldahl Distillation Apparatus.

The distillates are titrated with exactly $N/70$ HCl. The end-point is reached when the sample has the same color as the distilled blank. If the $N/70$ HCl is made up exactly, that is, with factor 1.000, calculation is extremely simple, since $\text{mg. N} = \text{ml. } N/70 \text{ HCl} \times 0.2$. The error in this procedure is about ± 0.01 mg N.

Notes. A run on a standard ammonium sulfate solution is useful to check the apparatus, procedure, and reagents. Blank runs on the reagents used for digestion should be performed and analyses corrected for any nitrogen in the reagents, or better reagents should be obtained.

If the distilled blank is faintly yellow (or green with alternative indicator) instead of faintly red (or pink), the distillation apparatus should be washed or steamed out until a proper blank is obtained. In this connection, the operator is cautioned against allowing the Kjeldahl flask to become more than $\frac{1}{3}$ or $\frac{1}{2}$ full during distillation. Otherwise, alkali may be splashed into the trap on the right (fig. 29) and the condenser. Improper color of the blank may also be due to impure indicator.

Addition of alkali *after* distillation has started will cause titration values to be low because CO_2 is steam distilled from the acid reaction mixture.

An apparatus similar to that in fig. 29, but using standard ground glass joints for the micro-Kjeldahl flasks is employed by Dr. W. F. Goebel.

MODIFICATION FOR SAMPLES OF 10-100 MICROGRAMS

The micro-Kjeldahl determination can be carried out using smaller samples without loss of precision with the distillation apparatus described by Markham (8), and illustrated in fig. 30, if titrations are performed with a micro-burette such as that described by Scholander (9), fig. 31. The same reagents are used as in the usual micro-Kjeldahl determination described above.

Procedure. 10 ml. Pyrex micro-Kjeldahl flasks with a lip for pouring are used for digestion. After introducing samples, add to each flask 1 boiling stone*, about $\frac{1}{4}$ gm. of K_2SO_4 and 0.5 ml. of concentrated H_2SO_4 saturated with CuSO_4 . Glass beads are inconvenient since they may fall into the distillation apparatus during transfer. Digestions are carried out as described for the usual method.

The apparatus shown in fig. 30 permits rapid quantitative distillation of ammonia in a small volume. Before use the apparatus should be steamed out for 5 minutes with clamps A and B closed. Clamps A and B are then opened and a blank is run by adding 3 ml.

* Manufactured by the Hengar Co., Philadelphia, Pa.

of saturated NaOH and about 20 ml. of water to the apparatus, closing clamps A and B and distilling about 12-15 ml. into a wide mouth test tube containing 1.5 ml. of solution D, prepared freshly each day by adding 0.1 ml. solution C to 30 ml. saturated boric acid. The alternative indicator mixture should be used with this method since it provides a more sensitive endpoint, especially when freshly prepared. The tip of the condenser should not be immersed in the distillate. At the end of the distillation, the flame is removed. This creates a partial vacuum which sucks the contents of the inner distilling flask into the outer steam jacket. Several portions of distilled water are introduced slowly through C for rinsing and are removed by back suction. The pinch clamp B leading to the drain is then opened and the solution removed from the apparatus. After washing the tip of the condenser, the tube of distillate is removed and replaced by another tube containing 1.5 ml. of solution D. Clamp A is opened and the flame is placed near the side of the boiling flask to prevent cooling. The digested sample is cooled, diluted with water and transferred quantitatively into the apparatus. The stopper at C is now closed and 3 ml. of saturated NaOH solution are added with a pipette and allowed to enter by lifting the stopper at C slightly without admitting air, followed by several portions of water introduced in the same way. Distillation is then started by placing the flame under the boiler and closing the pinch clamps A and B. Distillations are carried out in succession following this procedure. Each distillation takes about 2 minutes.

Titration is carried out by immersing the tip of the micro-burette under the surface of the distillate. Stirring is effected by bubbling a stream of nitrogen through the solution while adding N/70 HCl from the micro-burette. The nitrogen is washed by passage through a bottle containing boric acid solution. Each distillate is titrated to the color of the blank. The nitrogen content of the reagents used should be estimated frequently and any value deducted. Blanks should not exceed 3-4 $\mu\text{g. N}$. Since the indicator changes most rapidly in the colorless to faintly pink range, the distillation blank may be adjusted to this color by addition of a little HCl (ca 0.01 ml.).

The error is about $\pm 2 \mu\text{g. N}$ with this apparatus.

The micro-burette devised by Scholander (9) is made from a commercial one-inch micrometer and a glass vessel which can be con-

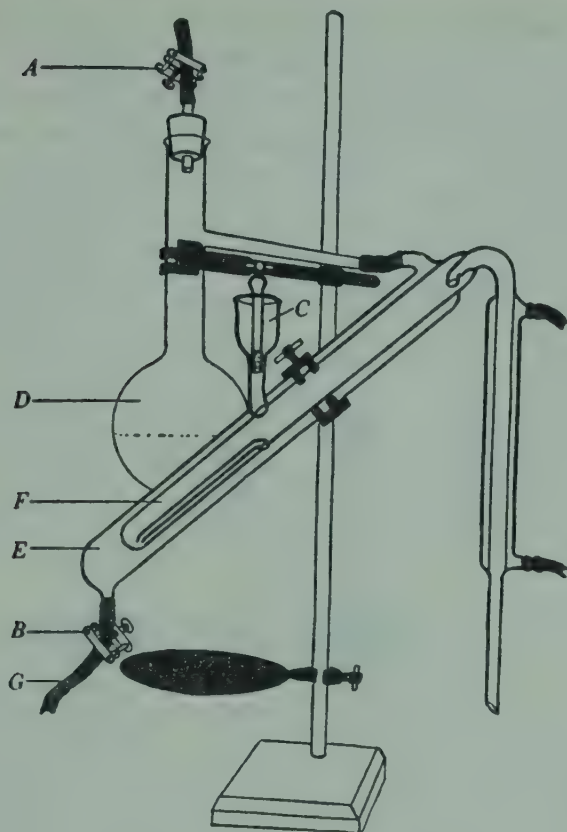


FIG. 30. MARKHAM KJELDAHL DISTILLATION APPARATUS

(The dimensions given below are those used by the authors; they depart somewhat from the proportions in the diagram.)

- A. Clamp on air-inlet tube. As used in the author's laboratories, the air-inlet tube extends to the bottom of the boiling flask. Clamp A is kept open during the distillation and closed only when back-suction is desired.
- B. Clamp for emptying drain.
- C. Cup for introducing digested samples and alkali. Diameter = 3.8 cm., height = 5 cm. Its position is near the upper end of the siphon tube. A well-fitted ground-glass plunger is used to close cup C.
- D. Boiling flask. In the author's laboratories, a 2 liter wide-mouth, round bottom or Erlenmeyer flask is used. An efficient vapor trap is placed in the steam line to prevent passage of liquid with the steam. The water in D is acidified with 2 ml. concentrated H_2SO_4 .
- E. Outer jacket. Length — 31 cm., diameter — 6 cm.
- F. Inner jacket (length = 31 cm., diameter = 4 cm.), containing a syphon tube (length = 19 cm., diameter — 0.8 cm.).
- G. Rubber tube to drain. The condenser is 40 cm. long (overall), and the diameter of the inner tube is 1.1 cm.

From (8). (The apparatus is obtainable from Mr. Frederick F. Anderson, 239 Greenwood Avenue, Madison, New Jersey.)

structed by a glass blower. The latter has a cylindrical chamber which is open on one side. A side arm of capillary tubing leads to the tip of the burette via a reservoir (fig. 31). The cylindrical chamber is placed in the micrometer frame with the open end toward the spindle. The latter displaces mercury which in turn pushes out the solution to be dispensed.

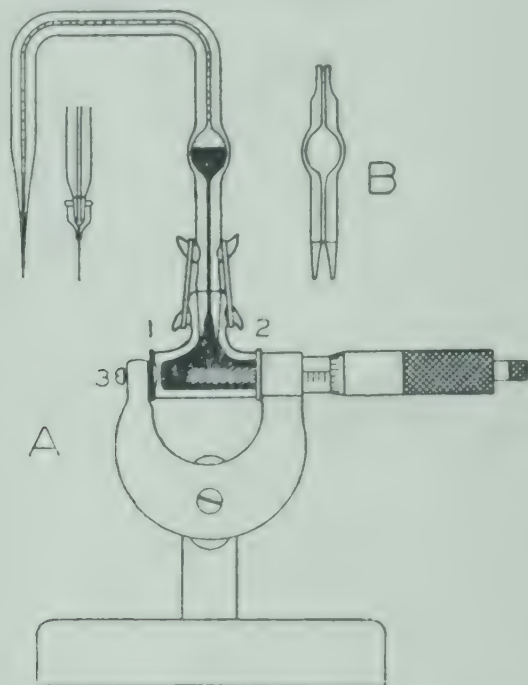


FIG. 31. Scholander Microburette. The length of the delivery arm of the burette should be twice as long as indicated. *From (9).*

Remove the anvil of the micrometer and replace it with a pointed screw (No. 3 in Fig. 31). Insert a hard metal disc (No. 1) with a center punch and a rubber disc between the point of the screw and the closed end of the cylindrical chamber. A neoprene washer (No. 2) making a close fit on the spindle is placed between the open end of the cylindrical chamber and the frame of the micrometer. The spindle is greased to prevent leakage of mercury.

After the glass chamber has been mounted in the micrometer, it is filled with dry mercury. Caution must be exercised to remove all air from the cylindrical chamber since the presence of air bubbles would reduce the accuracy of delivery.

The burette is calibrated by weighing successive portions of mercury delivered over the entire operating range. Most precise

delivery of mercury may be achieved by cutting off the drops of mercury at the tip with a razor blade (9).

For use in titrations the spindle is screwed in as far as possible, the tip is immersed in the titration fluid ($N/70$ HCl) and the spindle is retracted to the one-inch mark by turning the thimble. The tip of the burette is wiped and the instrument is ready for use.

The accuracy of this type of burette is quite adequate for the titration of 10 to 100 μ g. N. A special advantage of this device is the great speed with which portions of liquid may be delivered. No time need be allowed for drainage since the volume of fluid delivered depends solely on the volume of mercury displaced by the spindle.

For various purposes different types of tips may be needed. By means of a ground-glass joint a variety of tips may be attached to a single burette as desired.

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CHAPTER 13

PHOSPHORUS ESTIMATION

Micro-estimation of phosphorus may be carried out by many methods. A gravimetric and a colorimetric method are given.

Gravimetric Method: (Pregl-Lieb 1, 2, 3): Organic phosphorus is converted to inorganic phosphate by digestion with a mixture of sulfuric and nitric acid. The phosphate is then precipitated as ammonium phosphomolybdate, filtered, dried and weighed. Since the weight of ammonium phosphomolybdate is almost seventy times the amount of phosphorus, weighings may be made on an ordinary analytical balance with reasonable precision, when samples containing more than 0.5 mg. P are used. For smaller samples a semi-micro or micro balance is necessary and weighings should be made to ± 10 micrograms.

REAGENTS (after 2)

Molybdate Solution: Fifty grams of ammonium sulfate is placed in a 1-liter volumetric flask and dissolved in 500 ml. of concentrated nitric acid: 150 gm. of ammonium molybdate is dissolved in 400 ml. of boiling distilled water, cooled to room temperature and then poured slowly, with constant stirring, into the ammonium sulfate-nitric acid solution. The solution is then made up to the mark with distilled water, allowed to stand for several days and filtered into a glass-stoppered brown reagent bottle. It should be kept in a dark, cool place.

Nitric Acid-Sulfuric Acid Mixture: Thirty ml. of concentrated sulfuric acid (spec. gr. 1.84) is added to 1 liter of nitric acid of spec. gr. 1.19-1.21 (containing about 32 per cent nitric acid by weight).

Aqueous Solution of Ammonium Nitrate (2 per cent): The solution should be slightly acid; 1 drop of concentrated nitric acid may be added per liter of solution, if necessary.

Ethyl Alcohol (95 per cent).

Ether: Free from alcohol and water.

Acetone: Reagent grade free from aldehydes.

PROCEDURE (modified from 2)

Digestion: A sample of substance containing 0.1 to 0.5 mg. of P

(or from 0.5 to 2.0 mg. if an ordinary analytical balance is used for weighing) is placed in a clean micro-Kjeldahl flask; 0.5 ml. of concentrated sulfuric acid and 4 or 5 drops of concentrated nitric acid are added and the mixture is heated over a small flame until sulfur trioxide fumes are evolved. Addition of a few drops of nitric acid and boiling until SO_3 fumes are evolved is repeated twice. If, after cooling, the solution is still turbid, 4 or 5 drops of perhydrol are added and the material heated again until sulfur trioxide fumes appear. This is repeated until the solution is clear. The contents of the flask are rinsed into a wide test tube, 2 ml. of the nitric-sulfuric acid mixture added, and the volume is brought to 15 ml.

Precipitation: The test tube containing the solution is heated in a boiling water bath for about 10 minutes. Then 15 ml. of the freshly filtered molybdate reagent is run into it from a pipette without touching the side wall of the test tube. After 2 or 3 minutes, the mixture is shaken thoroughly and allowed to stand for at least 2 days. After completion of precipitation the tubes must not be heated again, because precipitation of molybdic acid may occur.

Filtration: The precipitates are filtered on a Pregl filter tube (fig. 32) on which a 2-3 mm. asbestos mat similar to those used on

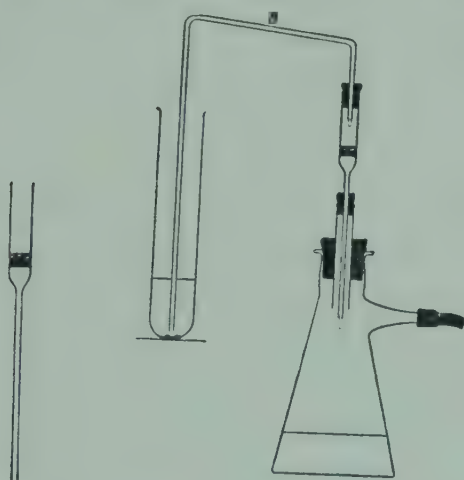


FIG. 32. Pregl filter tube. From (2)

Gooch crucibles has been placed. Before the filtration, the mat is washed with water, hot dilute nitric acid and distilled water; residual water is removed by rinsing twice with alcohol and twice with acetone or with ether. The filter tube is wiped with a moist flannel and dry chamois and placed in a desiccator, which is evacu-

ated on the water pump. No drying agents are used in the desiccator. The filter tube is removed from the desiccator after $1\frac{1}{2}$ hour and weighed. The procedure for quantitative transfer of the precipitate to the filter tube is as follows: The supernatant liquid is siphoned off, through the arrangement shown in fig. 32 and the precipitate is washed thoroughly in the test tube with 2 per cent ammonium nitrate solution and transferred to the filter tube. To remove the last traces of precipitate the walls of the test tube are alternately rinsed with ammonium nitrate solution and 95 per cent ethyl alcohol. The siphon is then removed and the filter tube is washed with alcohol and then with acetone or with ether. After wiping, the filter tube is placed in the desiccator which is evacuated. After 30 minutes, it is removed and weighed. The difference between the two weights gives the weight of ammonium phosphomolybdate.

Calculation:

$$\text{mg. P} = \text{mg. ammonium phosphomolybdate} \times 0.01443$$

$$\% \text{P in substance} = \frac{\text{mg P}}{\text{mg sample}} \times 100$$

Notes: A standard solution of KH_2PO_4 in 0.1 N H_2SO_4 may be employed to check the method. The factor, 0.01443 is used by Mr. William Saschek at the College of Physicians and Surgeons. Other workers (2, 3) use the factor 0.014525.

COLORIMETRIC DETERMINATION OF PHOSPHORUS BY THE FISKE-SUBBAROW METHOD (4)

The method is based on the development of a blue color when phosphomolybdic acid is reduced by 1, 2, 4 amino-naphthol sulfonic acid in the presence of sulfite. For uniform color development it is essential that the acidity of the solution be relatively constant. The standard conditions for colorimetric analysis are such that the solution is made up to contain 0.4 mg. of phosphorus in a volume of 100 ml. containing 0.25 gm. ammonium molybdate, 10 ml. 5N H_2SO_4 , 0.6 gm. of sodium bisulfite, and 0.01 gm. of 1, 2, 4 amino-naphthol sulfonic acid. Under these conditions, maximum color development occurs within 5-10 minutes. For estimation of inorganic phosphate in solutions containing protein, the protein is removed by addition of 4 volumes of 10 per cent trichloroacetic

acid. The acidity of the trichloroacetic acid must be taken into account by the use of a smaller amount of H_2SO_4 . This is done by preparing molybdate solution in varying strengths of H_2SO_4 .

REAGENTS

10 N Sulfuric Acid: 450 ml. of concentrated sulfuric acid added to 1300 ml. of water.

Molybdate I: 2.5 per cent ammonium molybdate in 5 N sulfuric acid. Dissolve 25 gm. of the salt in 200 ml. of water. Rinse into a liter volumetric flask containing 500 ml. of 10 N sulfuric acid. Dilute to the mark with water and mix.

Molybdate II: 2.5 per cent ammonium molybdate in 3 N sulfuric acid. Prepared as above, but with only 300 ml. of 10 N sulfuric acid. (To be used in the determination of inorganic phosphate after precipitation of protein with 4 volumes of 10 per cent trichloroacetic acid.)

Molybdate III: 2.5 per cent ammonium molybdate in water. As soon as any considerable amount of sediment (ammonium trimolybdate) has appeared in this solution, it should be discarded.

10 per cent Trichloroacetic Acid:

Standard Phosphate (5 ml. = 0.4 mg. P): Dissolve 0.3509 gm. of pure monopotassium phosphate in water. Transfer quantitatively to a liter volumetric flask, add 10 ml. of 10 N sulfuric acid, dilute to the mark, and mix. The standard keeps indefinitely.

15 per cent Sodium Bisulfite: The solution must be free from turbidity before it can be used. Freshly prepared sodium bisulfite solutions may not filter clear, in which case 2 or 3 days standing (before filtering) will be necessary. Keep well stoppered.

20 per cent Sodium Sulfite: Because it is likely to be of better quality than the anhydrous salt, the crystalline sulfite ($\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$) is recommended (4). Dissolve 200 gm. of this in 380 ml. of water. Remove any suspended matter by filtration, and keep stoppered.

0.25 per cent 1, 2, 4, Aminonaphtholsulfonic Acid: Dissolve 0.5 gm. of the dry powder (Eastman) in 195 ml. of 15 per cent sodium bisulfite, add 5 ml. of 20 per cent sodium sulfite, stopper, and shake until dissolved. If the bisulfite solution is old, more than 5 ml. of sulfite will be needed—in that event add more sulfite 1 ml. at a time, shaking after each addition, until solution is complete.

This reagent can be prepared in a few minutes, (the powder need not be very accurately weighed), and if not left exposed to the air it should keep about 2 weeks. The solution is more stable the higher its acidity, hence no more sulfite should be added than is needed to dissolve the reducing agent.

PROCEDURE

Determination of inorganic phosphate: The original directions of Fiske and Subbarow (4) called for making up all solutions to 100 ml. However, for ordinary colorimetric estimations 10 ml. or 25 ml. volumes are adequate and permit the size of the sample to be reduced. The directions given are for a total volume of 10 ml. except for determination of total phosphorus. For larger volumes, amounts of all reagents should be increased proportionately.

If no protein is present, the sample containing 0.02 to 0.08 mg. of inorganic phosphorus is measured into a 10 ml. volumetric flask. Add water to 7 ml. followed by 1 ml. of molybdate I, and 0.4 ml. 0.25 per cent amino naphtholsulfonic acid. After addition of each reagent, the solution should be mixed by gentle shaking. At the same time 0.5 ml. of standard phosphate solution containing 0.04 mg. of P is treated in the same manner. The contents of each flask are diluted to the mark, mixed, and compared in the visual colorimeter after 5 minutes. If a photoelectric colorimeter is used a calibration curve with varying amounts of standard solution should be prepared with a 6500 Å filter.

If protein is present 12 ml. of 10 per cent trichloroacetic acid is added to 3 ml. of the solution, the protein precipitate centrifuged off or filtered and 5 ml. aliquot portions of the filtrate treated as above except that molybdate II is added to compensate for the trichloroacetic acid present. The standard, however, is always made up with molybdate I and without trichloroacetic acid so that any variation in color intensity with time in the unknown solution may be detected by readings at varying time intervals after color development.

Determination of total phosphorus: The sample containing 0.1 to 0.4 mg. P is measured or weighed into a 100 ml. micro Kjeldahl flask or large Pyrex test tube. A glass bead is introduced to prevent bumping and 2.5 ml. of 10 N H_2SO_4 are added. The flask is heated on the micro-Kjeldahl digestion rack (see III, 12) until charring

occurs or fumes appear. Boiling is continued until no further blackening occurs and 1 drop of concentrated nitric acid is added. If the color does not disappear another drop of concentrated nitric acid is added until no color remains. Boiling is then continued until brown fumes no longer are formed.

The flasks are cooled and the contents of each flask rinsed into a 50 ml. volumetric flask with 35 ml. of water. 5 ml. of molybdate III and 2 ml. of the 0.25 per cent aminonaphtholsulfonic acid are added. Dilute to mark and read against the standard prepared as above.

Other methods: The Bodansky method for the determination of inorganic phosphate employs reduction with stannous chloride (5). A colorimetric method for total P (organic and inorganic) is that of King (6). A micro-method combining some of the features of the methods of Kuttner and Cohen (7), Fiske and Subbarow (4) and Benedict and Theis (8), has been described by Horecker, Ma and Haas (9).

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CHAPTER 14

AMINO NITROGEN DETERMINATION

The Van Slyke method for amino nitrogen (1-4) is based on the reaction between primary amines and nitrous acid resulting in the liberation of nitrogen gas:



Under the conditions employed by Van Slyke (5) all amino acids from proteins yield 1 atom of nitrogen per molecule of amino acid except lysine which yields 2 atoms and proline and oxyproline, which do not react to liberate nitrogen (5). The indole ring nitrogen in tryptophane and the imidazole ring nitrogen in histidine also do not react (5).

Certain amino acids, such as glycine and cystine, give somewhat more than the theoretical yield of nitrogen. The use of KI in acetic acid has been advocated (6) to obtain theoretical values with these substances.

Amino groups adjacent to carboxyl groups, as in α -amino acids react quantitatively in a few minutes at room temperature. For example, leucine yields the theoretical amount of N_2 in 3 min. at 22-24° C. (2). The minimum time required for quantitative evolution of nitrogen from α -amino acids with thorough shaking in the microapparatus is 4 to 5 min. at 15-20° C., 3 min. at 20-25° C., and 2 to 2½ minutes at 25-30° C. (3). With lysine, which contains an ϵ -amino in addition to the α -amino group, complete reaction requires about 15 min. at 25-27° C. (2), and about 30 min. at 20° C. (5).

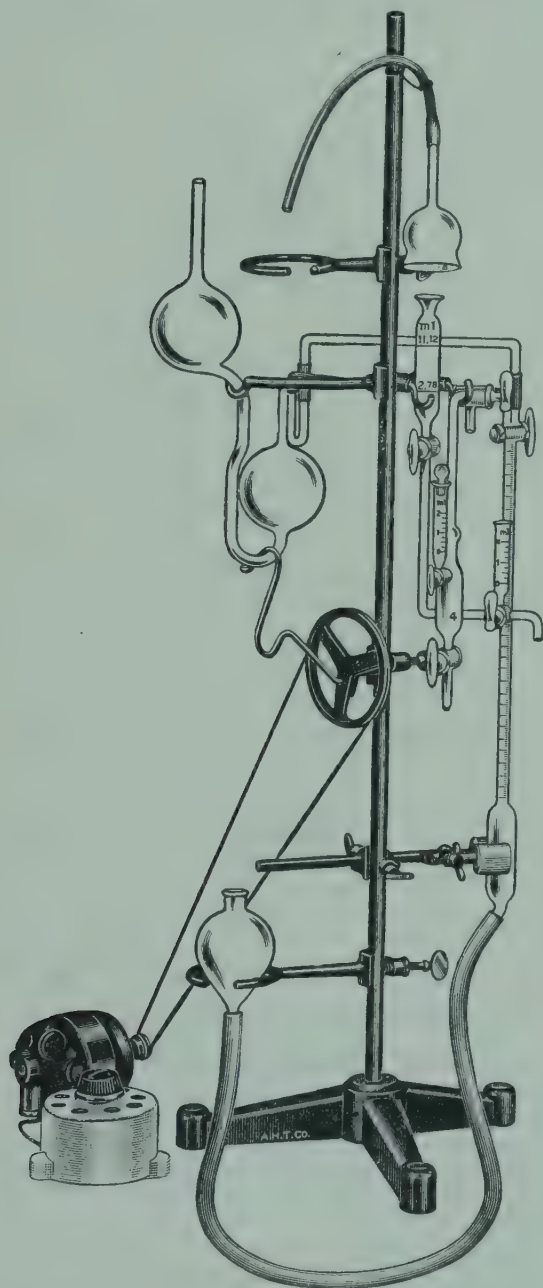
Ammonia also reacts but more slowly. Van Slyke's data (2) indicate 21.6, 36.3 and 62.1 per cent reaction in 3, 5 and 10 min. at 24° C. It is therefore necessary to remove ammonia from protein digests before determination of amino nitrogen. Complete reaction of ammonia or methylamine requires 1 to 1½ hours at 20° C. (5), while purines and pyrimidines react quantitatively in 2 to 5 hours. Urea requires about 8 hours (5). The guanidine group of arginine reacts very slowly. About 6 hours are required for liberation of an amount of N_2 equivalent to one-half the total nitrogen content of arginine (7).

The degree of hydrolysis of a protein after treatment with acid,

alkali or proteolytic enzymes may be calculated from the formula:

$$\text{Per cent hydrolysis} = \frac{100 (A - A_0)}{(A_1 - A_0)}$$

where A is the amount of amino nitrogen found in the partly hydrolyzed digest, A_1 represents the quantity of amino nitrogen at com-



Courtesy of Arthur H. Thomas Company

FIG. 33. Van Slyke apparatus for volumetric determination of amino nitrogen. *From (2).*

plete hydrolysis, and A_0 is the amount of amino N in the unhydrolyzed protein.

The determination may be carried out in a gas analysis apparatus designed either for volumetric or manometric measurement of the nitrogen evolved. A typical assembly of the volumetric apparatus is shown in fig. 33. It consists of three parts, namely, the reaction vessel (fig. 34), usually of 15 ml. capacity in the microapparatus,

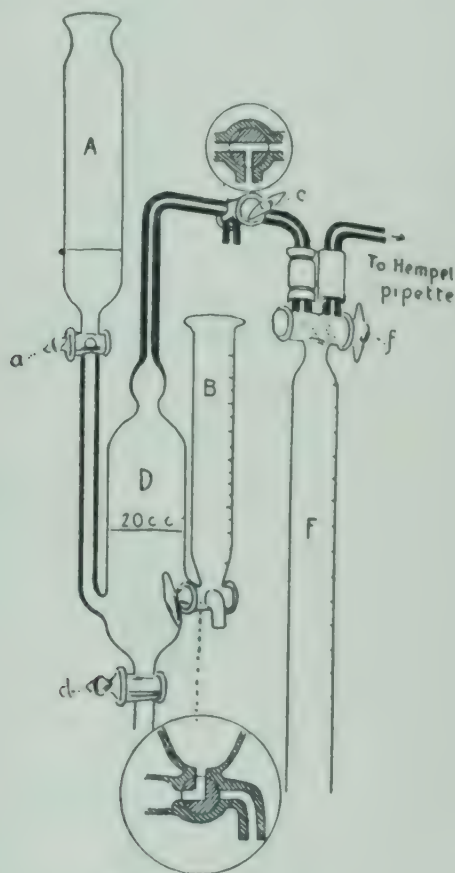


FIG. 34. Deaminizing bulb and connections, in detail. *From (2).*

the gas burette which holds 3 ml. and is graduated in subdivisions of 0.01 ml., and the absorption chamber (Hempel pipette shown at upper left, fig. 33). The latter contains an alkaline solution of potassium permanganate which serves to remove the nitric oxide (NO) formed in the decomposition of excess nitrous acid according to the reaction.



An improved design of the apparatus has been described by Koch (8).

Reagents: 1) 30 gm. NaNO_2 dissolved in 100 ml. of water. Let stand for several days before use. 2) Glacial acetic acid. 3) 50 gm. KMnO_4 and 25 gm. KOH dissolved in water and made to 1 liter. 4) octyl alcohol.

The procedure involves three steps. First, glacial acetic acid and sodium nitrite are introduced into vessel D and shaken to replace air by NO . The sample is then introduced, vessel D is connected with the burette, F, and the apparatus is shaken to facilitate complete evolution of gas. Finally, all the gases are driven into the alkaline permanganate solution where NO is removed, leaving the N_2 , which is then returned to the burette and measured. The sequence of operations, in detail, is as follows:

At the start of an experiment the levelling bulb, the burette, and the connections up to stopcock *c* are filled with water. There should be no gas in the connections between the burette and the Hempel pipette. Stopcocks *a*, *b* (at vessel B in Fig. 34) *d* and *f* are closed. Stopcock *c* is open to the drain (—). The levelling bulb is in the high position. Fill vessel A to the lower mark with glacial acetic acid (about 3 ml. in the micro-apparatus) this equals $1/5$ the volume of vessel D. Open *a* so that the acid runs into D. Close *a* and fill A to the upper mark with NaNO_2 solution (about 13 ml. in the micro-apparatus). Open *a* so that the nitrite solution enters D and turn *c* to position \perp when D is full. Since the combined volume of glacial acetic acid and sodium nitrite solution is somewhat greater than the capacity of D, there is enough liquid to fill D as well as the tube leading up to A. Shake the reaction vessel to facilitate evolution of NO gas. During this process the solution rises back into A. When D is about $3/4$ full of gas, i.e., when the liquid level has fallen to the mark (4 ml. in the micro-apparatus), stop shaking and open *c* to the drain (—) to allow the NO gas to escape. When D is again full of liquid, close *c* (\perp) and resume shaking. Again let NO out through *c* (in position —), when the level of liquid in D reaches the mark. Then close *c* (\perp) and repeat shaking. When the liquid in D falls again to the mark, close *a*, lower the levelling bulb at once and open *f* so that D is connected with the burette. Add the sample through B, being careful to accomplish a quantitative transfer without admitting air to D. If the substance is likely to foam, add some octyl alcohol either through B or by way of a special octyl alcohol inlet incorporated in some types of the apparatus. Shake vigorously

(as fast as the eye can follow) for the time required. Stop shaking and open *a* to drive gas into F. Close *c* and *f* when the liquid reaches *f*. Raise levelling bulb and open *f* to the Hempel pipette containing alkaline permanganate. When all the gas is driven over, close *f* and shake the Hempel pipette gently (not faster than twice every second) for 2 minutes. Drive the residual gas back into F, using the levelling bulb, until the permanganate solution is exactly at the zero mark of the burette (fig. 35). Close *f* and read the volume of gas while

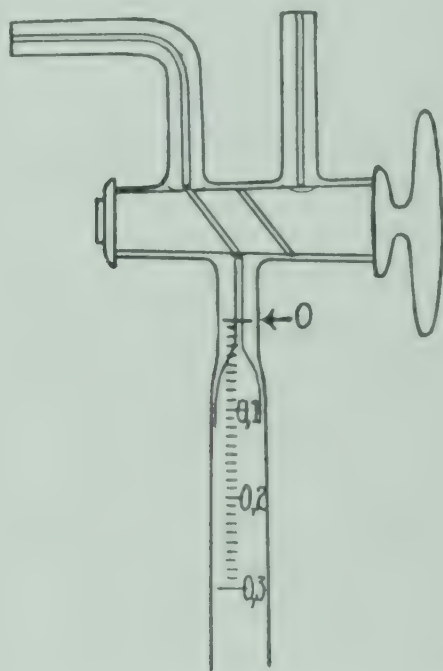


FIG. 35. Details of burette. From (4).

holding the levelling bulb at the same level as the water meniscus in the burette so that the gas is at atmospheric pressure. Then drive the gas back into the permanganate solution for another 2 minutes' shaking. Read the gas volume again at atmospheric pressure. Repeat the permanganate shaking until readings become constant. (This should not require more than 2 or 3 absorptions; otherwise, the permanganate is too old). To verify completeness of reaction, a second period of shaking may follow measurement of the N_2 gas evolved during the initial period of reaction. At the end of an experiment, drive the permanganate back into the Hempel pipette, since it would make stopcock *f* stick, remove the N_2 through *c*, and wash out the entire apparatus with water.

Every experiment is accompanied by a blank run with water instead of the sample. The volume of gas obtained in this control run, which should be about 0.06 to 0.12 ml. according to the quality and amount of nitrite employed, is subtracted from the volume obtained in the actual determination.

Take the temperature and barometric pressure and obtain the factor for converting the net (determination minus blank) ml. of N_2 into mg. of amino nitrogen from table 1. The values in table 1

TABLE 1

Milligrams of Amino Nitrogen Corresponding to 1 cc. of Nitrogen Gas at 11–30° C.; 728–772 mm. Pressure

t	728	730	732	734	736	738	740	742	744	746	748	750	t
11°	0.5680	0.5695	0.5710	0.5725	0.5745	0.5760	0.5775	0.5790	0.5805	0.5820	0.5840	0.5855	11°
12°	0.5655	0.5670	0.5685	0.5700	0.5720	0.5735	0.5750	0.5765	0.5780	0.5795	0.5815	0.5830	12°
13°	0.5630	0.5645	0.5660	0.5675	0.5695	0.5710	0.5725	0.5740	0.5755	0.5770	0.5785	0.5805	13°
14°	0.5605	0.5620	0.5635	0.5650	0.5665	0.5680	0.5700	0.5715	0.5730	0.5745	0.5760	0.5775	14°
15°	0.5580	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	0.5685	0.5705	0.5720	0.5735	0.5750	15°
16°	0.5555	0.5570	0.5585	0.5600	0.5615	0.5630	0.5645	0.5660	0.5675	0.5690	0.5710	0.5725	16°
17°	0.5525	0.5540	0.5555	0.5575	0.5590	0.5605	0.5620	0.5635	0.5650	0.5665	0.5680	0.5695	17°
18°	0.5500	0.5515	0.5530	0.5545	0.5560	0.5580	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	18°
19°	0.5475	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	0.5630	0.5645	19°
20°	0.5445	0.5460	0.5475	0.5495	0.5510	0.5525	0.5540	0.5555	0.5570	0.5585	0.5600	0.5615	20°
21°	0.5420	0.5435	0.5450	0.5465	0.5480	0.5495	0.5510	0.5525	0.5540	0.5555	0.5575	0.5590	21°
22°	0.5395	0.5410	0.5425	0.5440	0.5455	0.5470	0.5485	0.5500	0.5515	0.5530	0.5545	0.5560	22°
23°	0.5365	0.5380	0.5395	0.5410	0.5425	0.5440	0.5455	0.5470	0.5485	0.5500	0.5515	0.5530	23°
24°	0.5335	0.5350	0.5365	0.5380	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	24°
25°	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	25°
26°	0.5260	0.5295	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	0.5400	0.5415	0.5430	0.5445	26°
27°	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	0.5400	0.5415	27°
28°	0.5220	0.5235	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	28°
29°	0.5195	0.5210	0.5220	0.5235	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	0.5340	0.5355	29°
30°	0.5160	0.5175	0.5190	0.5205	0.5220	0.5235	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	30°
t	728	730	732	734	736	738	740	742	744	746	748	750	t

t	752	754	756	758	760	762	764	766	768	770	772	t
11°	0.5870	0.5885	0.5900	0.5915	0.5935	0.5950	0.5965	0.5980	0.5995	0.6010	0.6030	11°
12°	0.5845	0.5860	0.5875	0.5890	0.5905	0.5925	0.5940	0.5955	0.5970	0.5985	0.6000	12°
13°	0.5820	0.5835	0.5850	0.5865	0.5880	0.5895	0.5910	0.5930	0.5945	0.5960	0.5975	13°
14°	0.5790	0.5805	0.5825	0.5840	0.5855	0.5870	0.5885	0.5900	0.5915	0.5935	0.5950	14°
15°	0.5765	0.5780	0.5795	0.5810	0.5830	0.5845	0.5860	0.5875	0.5890	0.5905	0.5920	15°
16°	0.5740	0.5755	0.5770	0.5785	0.5800	0.5815	0.5830	0.5850	0.5865	0.5880	0.5895	16°
17°	0.5710	0.5730	0.5745	0.5760	0.5775	0.5790	0.5805	0.5820	0.5835	0.5850	0.5865	17°
18°	0.5685	0.5700	0.5715	0.5730	0.5745	0.5765	0.5780	0.5795	0.5810	0.5825	0.5840	18°
19°	0.5660	0.5675	0.5690	0.5705	0.5720	0.5735	0.5750	0.5765	0.5780	0.5795	0.5810	19°
20°	0.5630	0.5645	0.5660	0.5675	0.5690	0.5705	0.5725	0.5740	0.5755	0.5770	0.5785	20°
21°	0.5605	0.5620	0.5635	0.5650	0.5665	0.5680	0.5695	0.5710	0.5725	0.5740	0.5755	21°
22°	0.5575	0.5590	0.5605	0.5620	0.5635	0.5650	0.5665	0.5680	0.5695	0.5715	0.5730	22°
23°	0.5545	0.5560	0.5575	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	0.5685	0.5700	23°
24°	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	24°
25°	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	0.5625	0.5640	25°
26°	0.5460	0.5475	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	26°
27°	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	27°
28°	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	0.5520	0.5535	0.5550	28°
29°	0.5370	0.5385	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	0.5520	29°
30°	0.5340	0.5355	0.5370	0.5385	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	0.5490	30°
t	752	754	756	758	760	762	764	766	768	770	772	t

From (2)

represent the weights of *amino* nitrogen in milligrams which correspond to 1 ml. of nitrogen gas measured over water at the temperatures and atmospheric pressures indicated. These figures actually correspond to one-half the weight of 1 ml. of N₂ since only half the nitrogen comes from the amino group (cf. equation [1]).

With the micro-apparatus the error of the determination should not exceed 0.005 to 0.01 mg. of N or about 1 per cent for samples containing from 0.5 to 1 mg. of amino N.

The apparatus can be cleaned most conveniently by filling it with dichromate-sulfuric acid cleaning mixture. At frequent intervals the stopcocks and rubber connections should be tested for leaks by subjecting the assembly to moderate suction or pressure. When new, the calibration of the burette should be checked by weighing measured volumes of water.

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CHAPTER 15

ACETYL DETERMINATION (1)

The sample containing 1 to 5 mg. of acetyl (CH_3CO) is hydrolyzed for 2 hours under reflux with 20 ml. of a 25 per cent aqueous solution of p-toluene sulfonic acid, in a bath of boiling saturated NaCl solution. It is then transferred quantitatively to a 100 ml. micro-Kjeldahl flask and the acetic acid steam-distilled off in the micro-Kjeldahl apparatus (see III, 12) using an auxiliary Bunsen burner under the Kjeldahl flask, but without any addition of alkali. Successive 100 ml. portions of distillate are collected in 125 ml. Erlenmeyer flasks. After each 100 ml. is collected, the flame under the large boiling flask should be removed until the contents of the trap return to the Kjeldahl flask to prevent the trap from becoming too full. Each portion of distillate is heated to boiling under reflux, while a stream of CO_2 -free air is bubbled through the solution to remove the CO_2 . The flasks are cooled while CO_2 -free air is still passing through the solution, and the acetic acid is titrated with N/70 NaOH* until pink to phenolphthalein. Successive 100 ml. portions of distillate are titrated until a constant blank is obtained. This blank is subtracted from each titration to give the volume of NaOH needed to neutralize the acetic acid. The sum of these titrations after subtraction of the blank is used to calculate the acetyl content, as follows:

$$\text{mg. acetyl} = \frac{\text{ml. N/70 NaOH} \times 43}{70}$$

* N/70 NaOH may be prepared by dilution from saturated NaOH solution, and standardized by titration with standard N/70 HCl using methyl red as an indicator.

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CHAPTER 16

NEUTRAL EQUIVALENT

The quantitative determination of carboxyl groups is carried out by titration of a measured amount of substance with N/70 NaOH solution using a suitable indicator. In general, the choice of the indicator depends on the strength (pK) of the acidic groups; the indicator should have a pK at least 2 units larger than that of the groups to be titrated. Unless this condition is fulfilled, the end point will not be sharp, since the substance will act as a buffer in the pH range where the indicator changes color. In the case of strongly acidic polysaccharides such as certain pneumococcus polysaccharides, methyl red ($pK = 5.1$), phenol red ($pK = 7.8$) or phenolphthalein ($pK = 9.7$) will serve as indicator; the latter has the advantage that its color may be discharged with acid after the titration has been completed, the solution made up to a known volume and used for measurement of the optical rotation. Another advantage of phenolphthalein is the fact that it contains no nitrogen, allowing the use of the solution for nitrogen determination as well. When a polysaccharide contains O-acetyl groups, which would hydrolyze in alkaline solution, phenolphthalein cannot be employed since its endpoint lies near pH 9; in such cases phenol red which gives an endpoint at pH 7 to 8 is preferable.

The N/70 NaOH solution is prepared by dilution with CO_2 -free water (boiled water kept in a flask closed with a soda-lime trap) from a saturated solution of NaOH. The latter is prepared by dissolving about 70 gm. of NaOH in sufficient water to make about 100 ml. of solution. On cooling the sodium carbonate which is usually present in solid NaOH, separates as an insoluble sediment and scum and may be removed by centrifugation. A portion of the clear, saturated NaOH solution is diluted about 1000-fold with CO_2 -free water to yield approximately N/70 NaOH. The latter is kept in a paraffined vessel equipped with a soda-lime trap and a siphon so that samples may be withdrawn without exposure to the atmosphere. A convenient arrangement consists in connecting the N/70 NaOH stock bottle directly to the inlet tube of a burette with a three-way stopcock. Addition of a small amount of $BaCl_2$ to the solution of alkali is advantageous since any carbonate which

may be present would be precipitated by barium ion. The N/70 NaOH solution should be standardized by titration with N/70 HCl on the same day it is used for a neutral equivalent determination.

The titration of the substance is carried out in a 25 or 50 ml. Erlenmeyer flask. The size of the sample depends, of course, on the magnitude of the neutral equivalent. In general, if the neutral equivalent is about 100 to 200, a 10 mg. sample should suffice. For substances whose neutral equivalent is about 1000 to 2000, samples of about 100 mg. should be used. The frequently observed practice of boiling the solution just before the endpoint is reached when phenolphthalein is used as the indicator is not generally applicable to biologically active materials, if the substance is subsequently to be used for other purposes.

Calculation:—The neutral equivalent, which is the equivalent weight of a substance per acidic group, equals the molecular weight divided by the number of acidic groups. When the latter quantity is unknown, the equivalent weight gives a minimum molecular weight. The value of the neutral equivalent is obtained by use of the formula:

$$\text{Neutral equivalent} = \frac{\text{weight of sample in milligrams}}{\text{ml. NaOH used} \cdot \text{normality of NaOH}}$$

CHAPTER 17

DETERMINATION OF REDUCING SUGARS ON HYDROLYSIS

The determination of the amount of reducing sugar liberated on acid hydrolysis is of importance in characterizing polysaccharides. The reducing group of the sugar reduces the ferricyanide to ferrocyanide. The remaining ferricyanide in acid solution oxidizes the potassium iodide to iodine which is then titrated with sodium thiosulfate. The difference between a blank of ferricyanide in the absence of sugar and the value in the presence of sugar is a measure of the reducing power. Samples estimated to contain 2-6 mg. of reducing sugar are hydrolyzed in 2 ml. of 1 to 4 *N* HCl or H₂SO₄ for 2, 4 and 6 hours in sealed tubes placed in a pot of boiling water. After cooling, the contents of each tube are neutralized, made up to 15 ml. and 5 ml. aliquots are taken for the Hagedorn-Jensen method (1):

The 5.0 ml. aliquots and 5.0 ml. of Solution A are mixed in a test tube 1" in diameter. The tubes are covered and heated in a boiling water bath for 15 minutes and then cooled in running water. 5 ml. of Solution B and 3 ml. of Solution C are added and the iodine is titrated with sodium thiosulfate (Solution E) in a 25 ml. burette with a few drops of starch as an indicator (Solution D). A water blank is titrated in the same manner. The difference between the blank and the determination gives the thiosulfate equivalent of the ferricyanide reduced by the unknown solution. A calibration curve is prepared using known amounts of recrystallized glucose.

Solution A:

Potassium ferricyanide 8.25 gm. (Recrystallized and dried at 50° C.)

Anhydrous sodium carbonate 10.6 gms.

Make up to one liter, keep in dark.

Solution B:

Potassium iodide

5 gm.

Zinc sulfate 7H₂O

10 gm.

Sodium chloride

50 gm.

water to

200 ml.

Filter to remove iodine.

Solution C:

5 ml. glacial acetic acid diluted to 100 ml.

Solution D:

1 gm. soluble starch in 100 ml. of saturated NaCl solution.

Solution E:

N/200 $\text{Na}_2\text{S}_2\text{O}_3$. 0.7 gm. sodium thiosulfate per 500 ml. water.

The solution may be standardized against N/200 KIO_3 (0.3566 gms. per 2 liters) by adding a few ml. of HCl and 20 mg. KI per 10 ml. of iodate solution and titrating.

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CHAPTER 18

ESTIMATION OF URONIC ACID

A number of immunologically active polysaccharides have been found to contain glucuronic or galacturonic acid (cf. IV, 51), so that their determination is frequently important to the immunochemist.

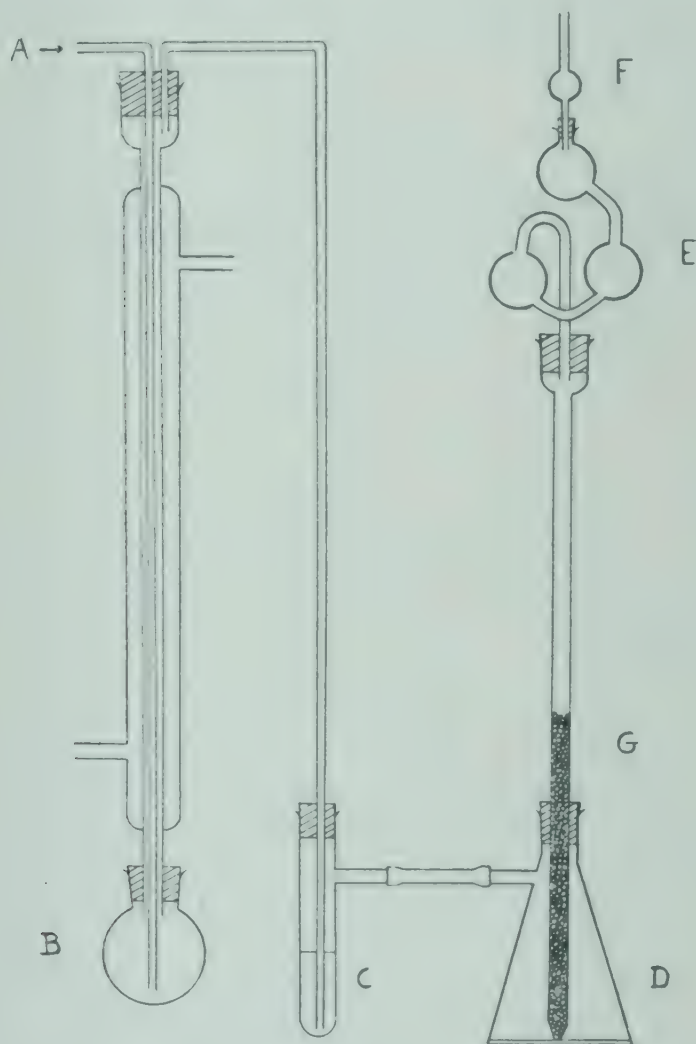
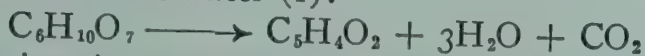


FIG. 36. Apparatus for the Estimation of Uronic Acid.

- A. Inlet for CO₂ - free air.
- B. Distillation flask of 25 ml. capacity.
- C. Test tube containing 5 ml. of a 5 per cent solution of AgNO₃.
- D. Suction flask containing a solution of Ba(OH)₂.
- E. Trap containing 1 ml. of Ba(OH)₂ solution.
- F. Soda-lime tube.
- G. Column packed with cracked glass.

Methods for the estimation of uronic acid are based on the fact that each molecule of uronic acid gives off one molecule of CO_2 quantitatively on distillation with 12 per cent HCl by weight and yields furfural and water (1):



The method given is a micro modification of the procedure described in (2). The sample containing 2 to 10 mg. of uronic acid is hydrolyzed with 20 ml. of 12 per cent HCl by weight under reflux while passing a stream of CO_2 free air through the solution and collecting the CO_2 liberated from the uronic acid in a volume of standard $\text{Ba}(\text{OH})_2$ (see fig. 36). After 6 hours hydrolysis the CO_2 liberated is determined by titrating the excess $\text{Ba}(\text{OH})_2$ with N/70 HCl until colorless to thymolphthalein. A blank is also run on the 12 per cent HCl alone. The HCl equivalent to the $\text{Ba}(\text{OH})_2$ neutralized by the CO_2 liberated is calculated and from this the uronic acid content of the sample is determined.

$$\text{mg. uronic acid} = 88 \times \frac{\text{ml. HCl equivalent to } \text{CO}_2 \text{ liberated (corrected for blank)}}{70}$$

A micro-method using the Clark micro methoxyl apparatus is also available (3).

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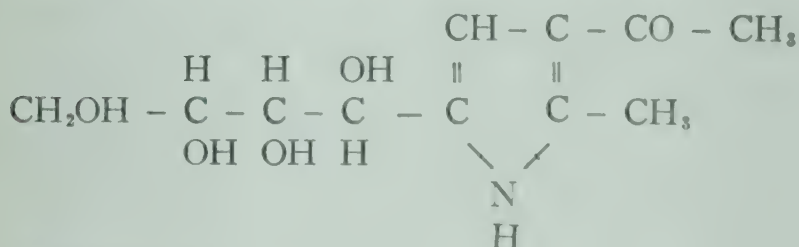
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CHAPTER 19

GLUCOSAMINE ESTIMATION

Many immunologically reactive polysaccharides have been found to contain amino sugar, notably glucosamine $\text{CH}_2\text{OH} \cdot \text{CHOH} \cdot \text{CHOH} \cdot \text{CHOH} \cdot \text{CHNH}_2 \cdot \text{CHO}$, and the determination of the hexosamine content is of value in the chemical characterization of these carbohydrates.

A modification (1) of the Elson and Morgan (2) method has been found to give reliable results even in the presence of protein. The method depends upon the color which develops when pyrroles are condensed with p-dimethylaminobenzaldehyde. Glucosamine reacts on heating in alkaline solution with acetylacetone to give 3 acetyl 2 methyl 5 tetrahydroxy butyl pyrrole (2, 3):



Reagents (1)

1. Acetylacetone solution, prepared immediately before use by dissolving 0.2 ml. of acetylacetone in 10 ml. of 0.5 N Na_2CO_3 . The acetylacetone (Eastman Kodak No. 1088) which should be colorless, is kept cold.

2. Aldehyde-free absolute alcohol (U. S. Industrial Chemicals).

3. p-Dimethylaminobenzaldehyde (can be obtained in pure form from Pfanstiel). Only pure white material should be employed.

Ehrlich's reagent is prepared by dissolving 0.8 gm. of p-dimethylaminobenzaldehyde in 30 ml. of aldehyde-free alcohol and adding 30 ml. of concentrated HCl. This solution may be kept in the refrigerator for a short period.

Procedure (4): Not all amino sugars are liberated from polysaccharides on hydrolysis with the same ease. It is therefore important to determine the hydrolytic conditions necessary to give maximum values by running hydrolysis curves, and then selecting

the mildest conditions which give maximum values for the substance under investigation.

A series of samples of the substance (to contain 25 to 125 μg of hexosamine, depending upon the thickness of the column of solution used in the photo-electric colorimeter) are weighed or measured into 13 x 100 mm. Pyrex test tubes and the volume made up to 1 ml. To one set of samples is added 1 ml. of 2*N* or 4*N* HCl, and to the other 1 ml. of ca. 8*N* HCl is added. The tubes are sealed off and are heated in boiling water for time intervals from 2-24 hours. The contents are cooled in ice water and carefully neutralized to phenolphthalein in the cold by the cautious addition of *N* NaOH or 4*N* NaOH, and the red color just discharged by the dropwise addition of *N* HCl. Extreme care must be taken to avoid local excesses of alkali during neutralization or low results will be obtained. It is recommended that practice determinations on known amounts of glucosamine in HCl be run to establish that losses of glucosamine are not occurring during neutralization. The solution is then transferred quantitatively to a 10 ml. volumetric flask and made up to mark.

In analyzing specific precipitates for glucosamine, the washed precipitate should be dissolved completely in 0.2 ml. concentrated HCl, 1.0 ml. distilled water added to make the acid concentration 2 *N*, the tube sealed and the solution hydrolyzed.

An aliquot of the neutralized hydrolysate (4 ml.) or of a solution containing a known amount of hexosamine is pipetted into a glass-stoppered 10 ml. Pyrex volumetric flask, and 1.0 ml. of freshly prepared acetylacetone solution is added from a 1.0 ml. analytical pipette. The flasks, held in a wire basket, are immersed in boiling water to a depth sufficient to cover the solution. After 15 minutes, the flasks are cooled by immersion in tap water. The contents are diluted with 3.0 ml. of aldehyde-free alcohol and 1.0 ml. of Ehrlich's reagent is added with stirring. Alcohol is then added to the mark and the flasks are shaken, care being taken to permit escape of CO_2 . Standard solutions, containing approximately 10 to 50 micrograms of glucosamine per ml., and a blank (4 ml. of water) are treated simultaneously in the same manner. The flasks are then placed in a water bath at 37 $^{\circ}\text{C}$. for 30 minutes. The light absorption of the test solutions and the standards is compared with that of the blank in a photoelectric colorimeter using a 5400 \AA filter. The

amount of hexosamine is calculated from the readings by means of the calibration curve prepared with solutions of glucosamine hydrochloride. The final colors developed from solutions of glucosamine and chondrosamine hydrochlorides are identical spectrophotometrically and of equal intensity for equal weights of the sugars.

Methods for the determination of N-acetylglucosamine have also been developed (5, 6, 7). By their use it may sometimes be possible to demonstrate the presence of N-acetyl glucosamine qualitatively after mild hydrolysis which avoids splitting the N-acetyl linkage.

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CHAPTER 20

CARBOHYDRATE ESTIMATION

In the estimation of carbohydrates in the presence of proteins methods of sugar analysis based on reduction (cf. III, 17) or optical rotation (cf. III-32) are not applicable since proteins possess reducing power and the capacity to rotate the plane of polarized light. However, a number of color reactions (1-7) such as those with α -naphthol (2), orcinol (3, 5, 6), phloroglucinol (2), diphenylamine (2), indole (1) and skatole (1) have been adapted for the determination of minute quantities of sugars in the presence of relatively large amounts of protein. While these reactions are specific either for carbohydrates in general, or for certain types of carbohydrates, such as hexoses or pentoses, they do not usually permit identification of individual sugars. Since different sugars generally do not react with the same color intensity, even when their structures are similar, it is necessary to ascertain the identity of the carbohydrate to be determined, so that analyses can be calibrated in terms of the sugar or sugars actually present. When the identity of the sugar is unknown, it is customary to express analyses in terms of a specified sugar arbitrarily chosen as a standard.

Unfortunately, none of the methods cited above can be recommended without qualification. The α -naphthol and the orcinol methods are given mainly because they have been used by the authors. In addition, the procedure of Dische for the determination of pentoses in the presence of hexoses is given.

The α -naphthol reaction: This is the familiar Molisch reaction adapted to quantitative measurement. The procedure is that described by Dische (2) as α -naphthol reaction I.

Reagents: 1) Sulfuric acid, diluted 8/9. Pour 80 ml. of concentrated H_2SO_4 (sp. gr. 1.84) into 10 ml. of water. Cool. 2) 5 per cent solution of α -naphthol in ethyl alcohol. The purification of α -naphthol may be readily accomplished by steam distillation and crystallization from toluol. The alcohol should be purified by treatment with pellets of NaOH followed by distillation. Prepare a fresh solution at least once a week and keep it cold and in the dark.

Procedure: Pipette 9 ml. of sulfuric acid (8/9) into a thin-walled 50 ml. centrifuge tube or large test tube. Immerse the tube in cold

water and add 1 ml. of sugar solution slowly from a pipette with constant mixing. Heat for exactly 3 minutes in boiling water. Cool to room temperature. Add 0.2 ml. of 5 per cent alcoholic α -naphthol solution and let stand at room temperature for ten minutes. Since α -naphthol and sulfuric acid produce a yellow-brown color which superimposes on the red-violet color given by the sugar, the color tints of analyses containing different amounts of sugar differ and the tubes cannot be matched in a colorimeter. Therefore, Dische (2) recommended that an α -naphthol-sulfuric acid blank be set up and used to dilute the stronger solution to the color intensity of the weaker one.

A procedure slightly different from Dische's has been used by one of the authors. 0.5 ml. of sample is mixed with 9 ml. of 8/9 sulfuric acid. After five minutes at 100° C., followed by cooling, 0.5 ml. of 1.3 per cent α -naphthol solution is added and the mixture warmed at 40° C. for 20 min. Readings are taken within the next 10 minutes with a spectrophotometer at 5750Å. Determinations and standards are read against a blank containing 0.5 ml. of water plus all the reagents.

In the analysis of proteins two additional blanks are needed: 1) protein solution plus sulfuric acid, and 2) sulfuric acid plus water. Blank 1 is read against blank 2 and the difference in optical density is subtracted from the density of the unknown read against the blank containing α -naphthol plus sulfuric acid.

Calibration analyses on standard sugar solutions should be run with every set of analyses.

Orcinol method according to (6):—0.5 ml. of sample is mixed with 2 ml. of 2 per cent orcin in 25 per cent (by volume) sulfuric acid and 15 ml. of 60 per cent sulfuric acid in weighted 150 ml. Erlenmeyer flasks covered with watch glasses. A standard amount of sugar is also set up. The mixtures are heated at 80° C. in the dark for 25 minutes (with 1 ml. samples 35 min. heating is better), and cooled in ice-water in the dark. Compare the color of the unknown with that of the standard in a colorimeter. It is necessary to carry out several determinations at different concentrations and to plot the amounts of sugar indicated against the amounts of protein used. The intercept of the line through these points with the zero protein coordinate is subtracted as a blank from all analyses.

Determination of pentoses in the presence of hexoses (7):—

Two procedures are described. I) 2 ml. samples of unknown and standard are each mixed with 2 ml. concentrated HCl containing 0.5 ml. of 10 per cent FeCl_3 solution per 100 ml. After addition of 0.2 ml. 10 per cent alcoholic orcin solution, the mixtures are heated for 3 min. in boiling water. II) Mix 1 ml. of sample and standard with 5 ml. of the HCl- FeCl_3 reagent and 0.4 ml. 10 per cent orcin solution. Heat for 3 min. in a water bath at 80°C . With both procedures, chill the mixtures in ice-water and keep in ice-water during the entire period necessary for photometry. Pentoses give a green color with both procedures as do nucleotides containing a pentose. Trioses also give this reaction but only at concentrations which do not normally occur in animal tissue. Hexoses give a yellow color. The color intensities are measured in a visual or photoelectric colorimeter using a 6100 Å filter. A 0.1 per cent solution of glucose gives no absorption, while a 0.01 per cent solution of fructose has about $1/20$ the absorption of an equivalent amount of adenylic acid in reaction I, and about $1/50$ the absorption in reaction II.

Methods for desoxyribose are given in III, 21.

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CHAPTER 21

NUCLEIC ACID DETERMINATION

Although nucleic acids are not known to show immunological properties, they are frequently present in products obtained from bacteria, tissues, etc., and their estimation may be of considerable interest. Determination of nucleic acid is also important in the study of bacterial and viral nucleoproteins.

The nucleic acids are high molecular weight polymers of tetranucleotides. A nucleotide consists of a nitrogenous base (purine or pyrimidine) linked to a sugar (ribose or desoxyribose) which is esterified with phosphoric acid. Each tetranucleotide consists of two purine and two pyrimidine nucleotides. Two general types of nucleic acids occur which differ chiefly in their carbohydrate moiety, and in their purine and pyrimidine bases, the yeast-type of nucleic acids containing ribose and uridine and the thymus-type containing desoxyribose and thymine in addition to adenine, cytidine and guanine. For details on the chemistry of nucleic acids, consult references (1-3).

Nucleic acids have the following composition based on the theoretical empirical formulae for their tetranucleotides (1-3).

	<i>Thymus type</i>	<i>Yeast type</i>
Empirical formula	$C_{39}H_{51}O_{25}N_{15}P_4$	$C_{38}H_{49}O_{29}N_{15}P_4$
Formula weight	1253	1303
Phosphorus %	9.89	9.52
Total Nitrogen %	16.76	16.12
Purine N %	11.17	10.74

• Methods for the quantitative determination of nucleic acids have been based on estimation of any of the three characteristic constituents—phosphorus, nitrogenous base or sugar. The first two of these yield values only for total nucleic acid, whereas procedures based on estimation of the sugar enable the type and amount of each nucleic acid to be determined.

Determination of the phosphorus content (III, 13) of samples containing protein may provide an estimate of nucleic acid content if other types of phosphorus such as lipid phosphorus or phosphate esters of hydroxyamino acids are absent.

The purines and pyrimidines of nucleic acids may be estimated by

their absorption of ultraviolet light, since they show a characteristic maximum at 2600 Å (III, 30) or by quantitative chemical analysis for purine N.

A micro-method for the determination of purine N developed by Graff and Maculla (4) requires samples containing about 0.5 mg. of purine N. The purines are precipitated with copper oxide after hydrolysis of the sample with *N* HCl in 10 *N* formic acid and analyzed by the micro-Kjeldahl (III, 12) method. Details may be found in (4).

Several color reactions for carbohydrates have been applied to the quantitative estimation of thymus nucleic acids (5-9). Of these the Feulgen (5) and carbazole (6) reactions are not specific for desoxyribose, the former being given by aldehydes and the latter by numerous other carbohydrates. Another recently developed method involves production of a red color with perchloric acid and tryptophane, but the reaction is given by other sugars (7).

One of the most specific reactions for desoxyribose is that of Dische using diphenylamine (8). The reaction yields a blue color if a suitable ratio (1 + 39) of sulfuric acid to glacial acetic acid is used. When higher concentrations of H_2SO_4 are employed, the reaction becomes less specific (8). Under the conditions employed all procedures except that involving perchloric acid and tryptophane (7) yield a color only with carbohydrate linked to purine.

Procedure (after 8):

Reagents: A. Diphenylamine solution: 1 gram of diphenylamine is dissolved in 100 ml. glacial acetic acid and 2.75 ml. concentrated H_2SO_4 (Spec. gr. 1.84) is added.

B. Blank solution: 100 ml. glacial acetic acid plus 2.75 ml. concentrated H_2SO_4 .

Procedure: To 4 ml. of the solution to be analyzed add 8 ml. of diphenylamine reagent (A) and to a second portion add 8 ml. of the blank solution (B). A second blank tube (C) containing water and solution (A) is also set up. Heat for 3 minutes in a boiling water bath, cool and after 10 minutes read the determinations and one of the blanks with a 6600 Å filter against the blank with least color. In this manner, the final value (the difference between the readings) is corrected both for the color of the protein in the acetic-sulfuric mixture and for any color of the diphenylamine itself.

A calibration curve may be prepared with known amounts of

sodium desoxyribonucleate using samples from 0.2 to 4 mg. The factor for nucleic acid from sodium desoxyribonucleate is 0.944.

The reaction is affected by buffer salts and protein breakdown products which cause a reduction in the acid concentration (8). In addition, proteins and other materials frequently are precipitated under the conditions of the reaction. Dounce (10) recommends centrifugation to remove any precipitate which may form.

Sevag, Lackman and Smolens (11) have studied the thymonucleic acid content of streptococci using the diphenylamine reaction. 25-35 mg. of dried ground whole organisms in 4 ml. of 0.1 *N* HCl are hydrolyzed in a boiling water bath for 15 minutes. 8 ml. of 1 per cent diphenylamine reagent are added (in 2 ml. of H_2SO_4 + 98 ml. of glacial acetic acid) and the estimation continued as outlined above. A blank is also run.

Another specific method for thymonucleic acid has been developed by Dische (9) which is based on the reaction of desoxyribose with sulphydryl compounds as follows:

Reagents: A. 5 per cent cysteine hydrochloride

B. 190 ml. water plus 450 ml. concentrated H_2SO_4 .

Procedure: To 1 ml. of solution (0.1 – 1 mg. thymonucleic acid) add 0.04 ml. solution A and 5 ml. solution B. Place in water bath at 40° C. for five minutes. A deep pink color with maximum absorption at 5000 Å develops and is read in the photoelectric colorimeter. Blanks without nucleic acid and cysteine are also set up. A calibration curve is obtained with known amounts of thymonucleic acid.

This reaction may be used even in the presence of large amounts of protein (8) which is kept in solution by the strong acid present.

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CHAPTER 22

ESTIMATION OF PROTEIN WITH THE FOLIN-CIOCALTEAU PHENOL REAGENT

Proteins may be estimated colorimetrically by measurement of the blue color produced by addition of the Folin-Ciocalteu phenol reagent to an alkaline solution of the protein. The color intensity produced by a given amount of a protein is chiefly a function of its tyrosine and tryptophane content, but other factors are known to play a role, especially the length of time to which the protein is exposed to alkali before addition of the phenol reagent. The analysis is performed directly on a solution of the protein without preliminary digestion. When equipment or time is limited, the method may be used in preference to the Kjeldahl, but initial calibration analyses by the Kjeldahl method are necessary if results are to be expressed in terms of nitrogen. The precision, 3-5%, is adequate for many purposes. A complete set of analyses may be performed within 2 hours. The usefulness of the method is, however, limited because only soluble proteins can be analyzed and because the color value of different proteins may vary widely and adequate results can be obtained only on single, well-defined proteins. Mixtures of several proteins of different chromogenic properties can be analyzed only if the constituents are invariably present in the same proportions. For example, the analysis of edema fluids or exudates is not advisable.

In each case a calibration curve must be prepared by the Folin method on solutions containing varying amounts of nitrogen as determined by Kjeldahl analysis. A linear relation is usually obtained in which case the color value of the protein per mg. of N can be calculated and used as a factor to convert optical density into mg. of N. Known solutions should be analyzed frequently as a check on the calibration. Serum albumin or tyrosine may be used as standards if the material under investigation is limited or unstable.

Reagents:

1. Folin-Ciocalteu phenol reagent (1).

Reagent grade chemicals of high purity should be used. Introduce 100 gm. $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, 25 gm. $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and 700 ml.

H₂O into a 1.5 l. or 2 l. flask with ground glass joint. Add 50 ml. 85 per cent H₃PO₄ and 100 ml. of conc. HCl. Attach a ground-glass joint condenser and reflux gently for 10 hours. Then add 150 gm. Li₂SO₄, 50 ml. H₂O and a few drops of liquid bromine. Boil without condenser for 15 minutes to remove excess bromine. Allow to cool, dilute to 1 l. and filter through paper into a thoroughly cleaned brown glass bottle. The reagent should be stored in the dark, protected from dust and other reducing agents. The bottle may be closed with a cork or rubber stopper but tin foil should not be used.

2. 20 per cent Na₂CO₃, anhydrous. Reagent grade.

Procedure: (For samples containing 100-500 μ g N)

Measure 9.0 ml. of sample in duplicate into a wide mouth 50 ml. centrifuge tube or a small Erlenmeyer flask. Add 5.0 ml. 20 per cent Na₂CO₃ slowly with constant mixing. Then add 1.0 ml. Folin reagent dropwise with *vigorous mixing*. Place in a 37° C. waterbath for 5 minutes. Let stand 30 minutes at room temperature, and read at 6500Å against a reagent blank, containing 9 ml. of water in place of the sample.

MICRO MODIFICATION FOR THE ESTIMATION OF 10-100 μ G. OF N

The method is made more sensitive by the addition of a minute amount of Cu⁺⁺ to increase color development (2), permitting the use of smaller samples. The best range of the method lies between 10 and 50 μ g. of N, using a colorimeter cuvette 13 mm. in depth. The error in repeated determinations is about ± 1 μ g.

Reagents:

1. Folin reagent.
2. 12.5 per cent solution of Na₂CO₃, anhydrous.
3. 0.1 per cent solution of CuSO₄·5H₂O.

Procedure (3):

Measure 2.0 ml. of protein solution into a tube or vessel sufficiently wide to permit efficient mixing. Add 6.0 ml. 12.5% Na₂CO₃ solution and 1.0 ml. of 0.1% CuSO₄ solution, mix and allow to stand for 1 hour at room temperature to ensure maximum color development. 1.0 ml. of $\frac{1}{3}$ Folin reagent (1 part freshly diluted with 2 parts of water) is then added slowly with *constant mixing*. After 20 to 30 minutes, the determinations may be read at 6500 Å against a blank of 2 ml. of water to which all the reagents have been added.

Special applications: The method has been used for the quanti-

tative micro-estimation of antibodies in the sera of man and other animals (3, 4, 5, 6, 7, 10). Using calibration curves with washed specific precipitates, obtained by addition of known amounts of antigen to a given volume of serum, this micro method may be used for the estimation of antigen in amounts as low as 0.5-10 μ g. N.

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CHAPTER 23

DETERMINATION OF PROTEIN CONCENTRATION BY DENSITY MEASUREMENT

It has been found that the density of plasma, serum or other protein solutions may serve as a reliable index of the protein content if the concentration of non-protein solutes is controlled (1, 2). Several rapid and precise densitometric technics have been developed which permit determinations to be made on a single drop of solution (3-6).

The method of Barbour and Hamilton (3) involves timing the fall of a standard-sized drop of protein solution through a definite distance in an organic liquid of low viscosity and immiscible with aqueous solutions. It has been found convenient to use xylene and bromobenzene mixed in a proportion to furnish a medium of somewhat lower density than that of the protein solution to be tested. The relation between the time of fall of a drop of known size at a given temperature and its density is found by calibration with standard solutions of potassium sulfate of known density. A direct application of this method to immunochemistry has been made by Nungester and Lewis (7) who determined total protein in anti-pneumococcus serum before and after precipitation of antibody with antigen, the difference being antibody protein precipitated.

The methods of Linderstrom-Lang and Lanz (4) and of Lowry and Hunter (6) utilize a density gradient, that is, a column containing two organic liquids of different densities layered over one another and partially mixed so that the density of the medium increases progressively from top to bottom. If a small drop of an aqueous solution is added just below the surface of such a gradient column, it will fall with decreasing speed and finally come to rest at an equilibrium position where the density of the surrounding medium is equal to its own. The density of the column at different heights is determined with drops of a series of standard K_2SO_4 solutions of known density. Since the character of the gradient changes with time due to diffusion, it is necessary to calibrate each time an unknown is to be analyzed. Although this represents a disadvantage, the gradient tube method (4, 6) is still more convenient than the falling drop

technic (3), since it does not require careful control of the size of drops.

While the gradient tube technic of Linderstrom-Lang and Lanz (4) is designed for extremely great accuracy to furnish density measurements with a precision of 3 parts per million, the much simpler modification of Lowry and Hunter (6), which yields density measurements with an accuracy of about 200 to 300 parts per million, suffices when an error of ± 0.1 per cent protein is permissible, as in the clinical determination of total serum protein.

The relation between the protein content of serum and its specific gravity* as determined with the gradient tube of Lowry and Hunter (6) is expressed by the linear equation

$$\text{per cent protein} = 348 \left(\text{Sp. gr.}_{20^{\circ} \text{C}}^{20^{\circ} \text{C}} - 1.0069 \right) \quad [1]$$

which differs slightly from the formula of Moore and Van Slyke (1),

$$\text{per cent protein} = 343 \left(\text{Sp. gr.}_{20^{\circ} \text{C}}^{20^{\circ} \text{C}} - 1.007 \right) \quad [2]$$

as well as from that of Weech et al (2)

$$\text{per cent protein} = 347.9 \left(\text{Sp. gr.}_{20^{\circ} \text{C}}^{20^{\circ} \text{C}} - 1.00726 \right) \quad [3]$$

The factors 348, 343 and 347.9 express the specific gravity contribution of the serum proteins while the values 1.0069, 1.007 and 1.00726 in equations [1], [2] and [3] represent the specific gravity of serum without protein. The accuracy of these equations is, of course, subject to the somewhat variable composition of the non-protein solutes, present in serum or plasma, that is, salts, N.P.N., etc.

In the analysis of purified proteins, however, it is possible to control the density or specific gravity contribution of non-protein solute by dialyzing against a solvent of known composition. With 5 x recrystallized horse serum albumin dialyzed against 0.9 per cent saline** (9.00 gm. in 1000 ml. of solution at 20° C.) the relation between protein concentration, measured by Kjeldahl analysis for nitrogen, and specific gravity, determined in the gradient tube (6), between 0.5 and 3 per cent protein content, follows the equation

$$\text{per cent protein} = 385 \left(\text{Sp. gr.}_{20^{\circ} \text{C}}^{20^{\circ} \text{C}} - 1.0065 \right) \quad [4]$$

where the value 1.0065 represents the sp. gr. $\frac{20^{\circ} \text{C}}{20^{\circ} \text{C}}$ which a solution containing no protein would have. Expressed in terms of density at 20°C ., equation [4] becomes

$$\text{per cent protein} = 385 (d^{20^{\circ} \text{C}} - 1.0047) \quad [5]$$

The small difference between the density of 0.9 per cent saline at 20°C (1.0046) as calculated by extrapolation from published data (8), and the value 1.0047 in equation [5] is probably due to experimental error.

A distinct advantage of density or specific gravity measurements as an index of protein concentration over other physical characteristics, such as optical rotation, refractive index or viscosity, lies in the fact that the specific density contributions (i.e., the factor 385 in equation [5]) of different proteins are often quite similar, so that a calibration curve on one protein may be applicable, at least approximately, to a number of other proteins. Thus Chick and Martin (9) found similar coefficients for crude horse serum globulin and albumin, the values being 357 and 337, respectively.

For rough assays ($\pm 0.1 \text{ mg. N}$) of the antibody content of strong sera, e.g. 2 mg. antibody N per ml., measurement of the decrease in density, after removal of the antibody with antigen, by the method of Lowry and Hunter (6) should prove satisfactory. With the more refined technic of Linderstrom-Lang and Lanz (4) precision will be limited by the accuracy of pipetting, but under favor-

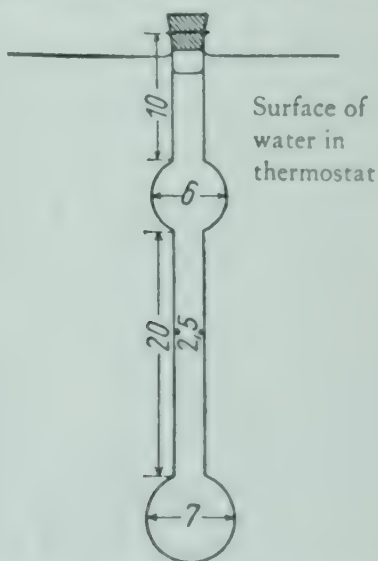


FIG. 37. The gradient tube. Dimensions in cm.

From (4).

able conditions may approach that of the more laborious analysis of washed specific precipitates for N by the micro-Kjeldahl method.

Procedure of Linderstrom-Lang and Lanz (4): A gradient tube of the shape and dimensions shown in fig. 37 is placed in a water-thermostat at $t^{\circ}\text{C.} \pm 0.002^{\circ}\text{C.}$ Two mixtures of white kerosene (sp. gr. 0.79) and c.p. bromobenzene (sp. gr. 1.48) are prepared with specific gravities of $(x + 0.01)$ and $(x - 0.01)$, respectively, where x represents the middle of the range of specific gravity to be covered. The gradient tube is filled to the middle of the connecting tube with the heavier of these mixtures. Using a funnel with a filter, the lighter mixture, previously brought to $t^{\circ}\text{C.}$ is then cautiously added up to level indicated in fig. 37. The density gradient is established in the connecting tube through partial mixing by gentle rotation and up and down motion of a long spatula. After 24 to 48 hours' standing, the gradient becomes so nearly linear that measurements may be carried out. Since water is not completely insoluble in the mixture of kerosene and bromobenzene, this medium must be saturated with an aqueous solution which has a vapor pressure approximately equal to that of the drops. 1 ml. of a solution of KBr (0.2 M for the density range 0.99 to 1.01) is shaken violently with 10 ml. of the lighter mixture, and the fine suspension is immediately poured into the gradient. The drops fall down through the tube, saturating it on the way. If the drops of KBr solution do not come to rest at the same level, repeat the saturation treatment.

If the length of the connecting tube is 20 cm., the smallest linear gradient possible will be $\frac{0.02}{20} = 0.001$. Since the position of a drop

in the column can be read with an accuracy of 0.001 to 0.002 cm. using a microscope, the maximal precision of density measurements is about 0.000002.

The gradient is calibrated with drops of KCl solutions of known density (cf. Landolt-Bornstein or International Critical tables). These standards are best prepared on a weight basis, that is, by dissolving a weighed quantity of KCl in a weighed amount of water. When not in use, they are stored at low temperature in volumetric flasks filled to the neck, and a layer of kerosene about 1 cm. thick is kept over the solution.

Drops of 0.1 to 0.15 mm³ are delivered from a small capillary

pipette with a constriction as a mark (10). The volume of a pipette is estimated by pipetting strong acid of known strength and titrating it with alkali delivered from a microburette (cf. III-12). The constriction pipette is filled by immersion in the standard solution below the layer of kerosene, rinsing several times and filling to the mark. The pipette is then raised to the level of the covering layer and about 0.1 mm³ of kerosene is drawn in. After withdrawal the tip is wiped with the edge of a piece of dry filter paper and about half of the kerosene is blown out into the filter paper. The pipette is now introduced into the gradient tube with the tip about 2 mm. below the surface, and the liquid is blown out gently without blowing air. The drop is removed from the tip when the pipette is withdrawn from the surface of the gradient column. It falls slowly and reaches its equilibrium position in about 15 min. After an experiment, drops are removed from the gradient tube with a long, thin glass rod with a sharp point to which the drops adhere so that they may be drawn up through the tube. At the surface they are attached to the wall of the tube. The glass rod should be inserted and withdrawn slowly and without shaking, so that the gradient is not unduly disturbed.

The equilibrium position of a drop is measured with a horizontal microscope A (fig. 38) of long focal length, pivoted at B to move in a perfectly horizontal plane. The height of the vertical column supporting the microscope may be adjusted roughly with a rack, C, of 10-15 cm span, and fine adjustments are made with the micrometer screw D. The whole apparatus is built on a triangular base supported by three levelling screws.

To measure the height of a drop the micrometer screw D is set at zero, and using the coarse adjustment, C, the cross hair in the eyepiece is set at the lower edge of a standard drop below the unknown drop. Clamp E is then tightened to fix the rack and by means of the micrometer screw, D, the microscope is raised until the cross hair coincides with the lower edge of the standard drop above the unknown drop. Screw D has a range of 10-12 mm, one revolution corresponding to 1 mm. The circumference is divided into 100 parts, allowing readings to be made with an accuracy of 0.01 mm. Finally the microscope is moved vertically by means of screw D until the cross hair is set at the lower edge of the unknown drop between the two standard drops and a reading is taken. If the gradient is linear

the density of the unknown drop, may be calculated by interpolation. The linearity of the gradient should be checked with a series of closely spaced standard drops.

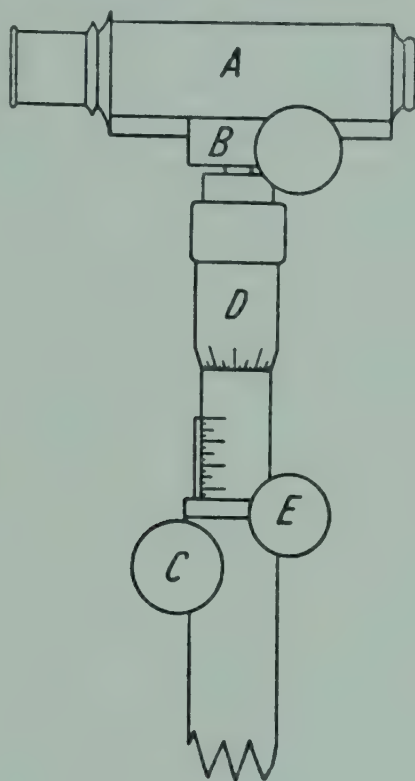


FIG. 38. The microscope. From (4.).

Procedure of Lowry and Hunter (6): The gradient column is contained in an ordinary 500 ml. measuring cylinder immersed in a slightly larger cylindrical glass jar, and held in a vertical position with a layer of paraffin wax around its base. The intervening space is filled with 0.2 per cent CuSO_4 solution in 0.1 per cent H_2SO_4 which serves to prevent convection currents by absorbing radiant heat, and a layer of paraffin wax is poured on top of the CuSO_4 solution.

The specific gravity range of the gradient is from 0.99 to 1.07 which serves for saline solutions containing up to about 15 per cent protein. The heavier solution (sp. gr. 1.07) is composed of 100 ml. of bromobenzene (sp. gr. 1.49) and 150 ml. of white kerosene (sp. gr. 0.80); the lighter mixture (sp. gr. 0.99) is prepared by mixing 70 ml. of bromobenzene with 180 ml. of kerosene. After filling half of the cylinder with the sp. gr. = 1.07 mixture the lighter fluid is added

slowly through a funnel with a filter paper, arranged so that the liquid runs down the wall. The gradient is now established by partially mixing the two layers with a long copper wire bent in a flat spiral at one end. Up and down strokes of uniform velocity are made centering about the initial position of the liquid boundary, with an initial amplitude of 7-8 cm. After about 20 strokes the amplitude is increased to about 15 cm. and finally strokes are extended throughout the entire height of the column, except for the last 5 cm. at the top and bottom. Drops of standard salt solution of known density are introduced to inspect the gradient. If they are spaced too closely for the range of operation desired, or if the gradient does not approximate linearity, more full-length strokes with the copper spiral are needed. If the standard drops are spaced too widely, there has been too much stirring and a new gradient should be prepared. The gradient is saturated with water by pouring an emulsion of 20 per cent aqueous NaCl and the lighter mixture into it. After standing for 1 day, the gradient is ready for use.

The gradient cylinder should be set on a level table free from vibration, direct sunlight and immediate sources of heat. Observation of the drops will be facilitated by indirect fluorescent (cool) lighting from the rear. With time the gradient becomes more nearly linear due to diffusion. After 3-6 months, however, the heaviest standard drop will fall to the bottom, and a new gradient has to be prepared. When not in use the gradient should be tightly covered to prevent evaporation of the toxic bromobenzene.

Drops of 2 to 4 mm³ volume are delivered from constriction pipettes like those used by Linderstrom-Lang and Lanz (4). These are made from pipette tubing drawn out into the shape of an ordinary dropping pipette about 12 cm. long. Using a hypodermic needle (gauge 22-25) as a microburner, a constriction is made about 6-8 mm. from the tip. Finally the tip is constricted as well. To permit discharge of 2 drops for duplicate readings on the same solution, Lowry and Hunter (6) recommend pipettes with two constrictions, one above the other. For use, the pipette is rinsed thoroughly about 5 times with the solution to be tested and each rinsing is discarded. It is then filled to the upper constriction, using a rubber tube with mouthpiece. The tip of the pipette is gently wiped on a filter paper before introduction into the gradient. With the tip slightly below the surface of the gradient, the solution is blown down to the middle

constriction where surface tension will hold it. By raising the tip through the surface of the gradient, the drop is detached and falls. The pipette is then reinserted and the second drop is delivered, taking care not to blow air as this might break the drop. The same pipette may be used for different solutions provided it is rinsed several times with each new solution.

Drops of suitable standard salt solutions are introduced before the protein drops, taking care to wipe the pipette and rinse it thoroughly with each new solution. The pipette used for salt solutions may subsequently be employed for protein solutions, but should be thoroughly rinsed. Readings are taken 4 minutes after introduction of the last drop in a series of experiments. A ring of stiff paper placed around the cylinder so that it can slide vertically will help avoid parallax since the position of a drop can be read by aligning the front and rear edge of the paper ring with the lower edge of the drop.

Readings of position of the standard drops are plotted on graph paper against specific gravities (N densities) or against the corresponding protein concentrations, and from the curve plotted through the points the concentration of the unknown is evaluated. Since the densities of protein solutions and salt standards have similar temperature coefficients within the precision of this method it is not necessary to consider the temperature of the gradient.

To remove droplets sprinkle some fine sand into the gradient. The sand particles adhere to the droplets and will carry them to the bottom. Another method consists in introducing a stirring rod, wrapped with a piece of wet filter paper to which the drops will adhere. This operation should be carried out as carefully as possible to avoid disturbing the gradient. When the gradient becomes cloudy with use, sprinkle a little CaCl_2 into it.

The specific gravity standards used by Lowry and Hunter for serum protein determination are prepared from c.p. K_2SO_4 dried overnight at $100-110^\circ \text{C}$. Exactly 17.64, 23.08, 28.53, 34.04, 39.58 and 45.10 gms. of K_2SO_4 are weighed out and dissolved in water up to exactly 1 liter at 20°C . to give 6 standards of sp. gr. $\frac{20^\circ \text{C}}{20^\circ \text{C}}$ equal 1.0141, 1.0184, 1.0227, 1.0270, 1.0313 and 1.0356, which are equivalent to serum protein concentrations of 2.5, 4.0, 5.5, 7.0, 8.5 and 10.0 per cent respectively. These standards are stored in the cold under a layer of kerosene. It is convenient to keep them in bottles equipped with a syphon, but the syphon outlets should be closed

with rubber plugs to prevent evaporation. For working standards 5 or 10 ml. amounts of each solution are kept in rubber-stoppered vials, but these should be emptied and refilled every week from the large samples in the refrigerator. If other standards are desired these may be prepared according to density data in Landolt-Bornstein or International Critical Tables.

- * It should be noted that density and specific gravity are not the same (8). Density is defined as the mass of unit volume of solution at temperature t . Its dimensions are grams per cc. or grams per ml. In precise measurements the difference between ml. and cc. should be noted, namely, $1 \text{ cc} = 0.999973 \text{ ml}$. At 3.98°C . the density of water equals 1.000000 gm./ml. or 0.999973 gm./cc.

Specific gravity equals the ratio of the mass of a certain volume of solution at temperature t to the mass of the same volume water, at the same temperature t , or some other specified temperature t' . Specific gravity is therefore stated as sp. gr. $\frac{t}{t}$ or as sp. gr. $\frac{t}{t'}$. If

$t' = 4^\circ \text{C}$., specific gravity equals density, i.e. sp. gr. $\frac{t}{4^\circ \text{C}} = d^t$.

Sp. gr. $\frac{t}{t}$ of a solution is calculated as follows:

$$\text{sp. gr. } \frac{t}{t} = \frac{W_1 - W_2}{W_3 - W_2}$$

where W_1 is the weight of a pycnometer filled with solution at temperature t , W_2 equals the weight of the pycnometer when empty, and W_3 represents its weight when filled with distilled water, also at temperature t . The specification of temperature refers to the temperature at which the pycnometer is filled to the mark.

To convert sp. gr. $\frac{t}{t}$ to sp. gr. $\frac{t}{4^\circ \text{C}} = d^t$, it is necessary to multiply by the density of water at t ,

For example, if the weight of a solution in a pycnometer filled at 20°C . equals 1.600 gms. ($W_1 - W_2$) and the weight of water in the same pycnometer, also filled at 20°C . is 1.2000 gms. ($W_3 - W_2$), the sp. gr. $\frac{20^\circ \text{C}}{20^\circ \text{C}}$ equals 1.3333 . Multiplying this value by 0.9982 , the density of water in gms. per cc. at 20°C ., yields the density 1.3315 gms/cc.

- ** The method of expressing the concentration of a solution is also an important matter. In tables of physical constants (8) concentrations are frequently stated on a weight per cent basis (P_w), that is the weight ratio of *solute* to *solution*, multiplied by 100. For example, a 1 per cent solution on a weight basis is made up from 1 gm. of *solute* and 99 gms. of *solvent*. When expressed in this way, it is not necessary to state temperature. However, if concentration is expressed as per cent on a volume basis (P_v), that is gms. of *solute* per 100 ml. of *solution*, the temperature at which the solution is made to volume should be stated. Another method, also requiring specification of temperature, consists in dissolving a given weight of *solute* in a stated volume of *solvent*.

The relation between weight per cent (P_w) and volume per cent (P_v) concentrations is given by the equation

$$P_v = P_w \cdot d_{\text{solution}}^{t^\circ \text{C}}$$

The relation between weight per cent (P_w) and concentration (C) expressed as weight of solute per volume (V) of solvent is given by the equation

$$P_w = \frac{C}{V \cdot d_{\text{solvent}}^{t^\circ \text{C}} + C}$$

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CHAPTER 24

ESTIMATION OF ENZYME ACTIVITY

For studies on the purification and chemical properties of enzymes, as well as for immunochemical studies on enzymes, it is essential that precise methods of measuring the concentration of enzyme be available. The determination of enzyme activity involves consideration of reaction kinetics. The rate of decomposition,

$\frac{dS}{dt}$, of a substrate by an enzyme often follows a zero-order equation

during the initial stages of the reaction, if the substrate concentration is so high that the enzyme works at maximal speed, i.e.

$$\frac{-dS}{dt} = k \quad [1]$$

Integration of equation [1] yields

$$S_0 - S = kt \quad [2]$$

where S_0 is the initial substrate concentration and S is the concentration at time t . At later stages or if the substrate concentration is low the kinetics of the reaction may be of the first order, i.e., follow the equation

$$\frac{-dS}{dt} = kS \quad [3]$$

which on integration yields

$$\ln \frac{S_0}{S} = kt \quad [4]$$

The constant k in equations [1] to [4], is known as the specific reaction rate.

The catalytic effect of enzyme may be considered as an increase in k and is proportional to the concentration of enzyme, i.e., $k \propto E$. Estimation of enzyme activity therefore involves determination of the specific reaction rate k as a measure of the enzyme activity. In zero order reactions, k equals the rate of substrate decomposition (cf. equation [1]), but with first order kinetics this is not the case.

Here the use of reaction velocity instead of k as a measure of enzyme concentration would yield erroneous results.

Instead of calculating k , it is often more convenient to employ the reciprocal of the time necessary to produce a given change in substrate concentration as a measure of enzyme activity. Solving equations [2] and [4] for k yields, respectively,

$$k = \frac{1}{t} (S_0 - S) \quad [5]$$

and

$$k = \frac{1}{t} \ln \frac{S_0}{S} \quad [6]$$

For a given amount of substrate decomposition $(S_0 - S)$ or $\ln \frac{S_0}{S}$ are

constant, and k is therefore inversely proportional to t . In practice, this method involves determining t for a given substrate change at two different enzyme concentrations (E_1 and E_2) so that the ratio,

$$k_1/k_2 = t_2/t_1 \quad [7]$$

is a measure of the relative enzyme concentrations employed, i.e., E_1/E_2 . This calculation is, of course, only valid if the time-action function is the same at both enzyme concentrations.

Three different methods of measuring enzyme concentration are thus available (1): (a) the amount of reactant changed in a given time during the initial stages of the reaction (zero order kinetics), (b) extrapolated or average values calculated as reaction constants, usually monomolecular reaction constants, and (c) reciprocals of the times required to effect a given change. Detailed theoretical consideration of the conditions under which each method is valid have been given by O. Bodansky (1) and by Van Slyke (2).

The first method using the amount of reactant changed in a given time during the first part of the reaction as a measure of the reaction velocity, is very widely used in enzyme studies, especially in clinical laboratories. Its use is justified only when the reaction has been shown to be of zero order for each enzyme concentration, i.e., follows equation [1]. The amount of substrate transformed in a given time is then independent of the initial substrate concentration and is a measure of the amount of enzyme. Hence, in a given time,

twice as much substrate will be split if the enzyme concentration is doubled.

For precise data using this method, each enzyme system should be studied to determine at what substrate concentration and over what portion of the initial range, if any, the reaction is actually of zero order. All measurements of enzyme concentration should be made within these limits. The range over which any given enzymatic reaction is of zero order may vary with the conditions under which the enzyme acts. Thus dialyzed solutions of bone or kidney phosphatases have been found to split glycerophosphate at a constant rate only during the initial 1 or 2 per cent hydrolysis. In the presence of optimal amounts of glycine and magnesium, the reaction remains of zero order until about 10 per cent of the glycerophosphate is hydrolyzed.

Bodansky (1) deplors the not uncommon practice of selecting an arbitrary time interval from the beginning of the reaction during which the amount changed is measured, regardless of the extent of the action which this amount represents and of whether the reaction is of zero order during this interval. The magnitude of the error introduced will depend upon the degree of deviation from a zero order reaction.

The second of these methods, i.e., calculation of the monomolecular reaction constants from equation [4] may be used with low substrate concentrations when the values for k as obtained from this relation are actually constant within experimental error during the course of the reaction, indicating that the reaction is of the first order. (Kinetics are those of a monomolecular reaction). Under these conditions, the values for k found will be directly proportional to the enzyme concentration and thus be a measure of it. When k is not constant over the reaction range investigated the assumption of a monomolecular reaction is unjustified and erroneous results may be obtained.

The third method—the use of the reciprocal of the time required to effect a given change as a measure of reaction velocity and hence of relative enzyme activity—is in many respects the most convenient for experimental work. It was first formulated for enzyme systems by Arrhenius (3) as the Qt rule (Q = enzyme concentration in this case): That the product of the time required for an enzyme to effect a given change in a substrate and the concentra-

tion of the enzyme was constant. This follows from [7] because $k_1t_1 = k_2t_2$, where k is a measure of enzyme concentration, E , so that $E_1t_1 = E_2t_2$. Bodansky (1) has demonstrated this relationship generally.

Table I (1) illustrates the use of the method with alkaline phos-

TABLE I

Effect of Accelerants on Value of Ratio of Reaction Time at 75 Per Cent by Volume Concentration of Phosphatase Extract to that at 12.5 Per Cent Concentration

Temperature 25°; concentration of substrate, sodium β glycerophosphate 0.0127 M; optimal pH 9.1; cattle bone phosphatase

P liberated as phosphate per ml. hydrolysis mixture	Time necessary for liberation of phosphate at		Ratio of reciprocals of reaction times
	12.5 per cent concentration of phosphatase	75 per cent concentration of phosphatase	

In presence of 0.00625 M glycine and 0.009 M magesium

mg.	min.	min.	
0.02	40	6.8	5.9
0.03	60	10.1	5.9
0.04	81	13.6	6.0
0.05	102	17.0	6.0
0.06	121	20.2	6.0
0.07	140	23.5	6.0

In absence of added accelerants

0.02	145	15.2	9.5
0.03	268	23.1	11.5
0.04	440	31.2	14.1
0.05	642	39.6	16.3
0.06	860	48.5	17.7
0.07	1135	57.6	19.7

phatase. In the presence of optimal amounts of magnesium and glycine, the ratio of the reciprocals of the times necessary for liberating from 0.02 to 0.07 mg. P with concentrations of 12.5 per cent and 75 per cent phosphatase is 6, i.e., proportional to enzyme concentration. However, in the absence of these accelerants, variations in the ratio of the same solutions from 9.5 to 19.7 are found when the amounts of P liberated were 0.02 to 0.07.

Failure to find a constant ratio indicates that the time-action function is different for the two enzyme concentrations because the concentration of α -amino acids (present as products of autolysis) is greater at the higher concentration of enzyme extract and hence

prevents, to a larger extent, the inactivation of the phosphatase during its action on the substrate.

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CHAPTER 25

ELECTROPHORETIC ANALYSIS

The fundamental observation that charged particles in solution migrate in an electric field has led to the development of one of the most powerful tools for characterizing proteins and numerous carbohydrates and has provided one of the few physicochemical criteria of protein homogeneity. Although a detailed treatment of the theoretical basis of electrophoresis is beyond the scope of this volume, it is essential that the immunochemist be familiar with the principles of the moving boundary method as used in the Tiselius electrophoresis apparatus (1, 2, 3) and be able to evaluate critically the data which appear in the literature.

If a protein solution buffered at any given pH is placed in a U-tube and pure buffer solution is carefully layered over it, the protein will migrate into the buffer toward one of the electrodes when direct current is passed through the solution. With a single protein, all the molecules will move at the same rate so that sharp boundaries between the protein and the buffer will be maintained. With a solution containing a single protein component, electrophoresis may be illustrated diagrammatically as shown in figure 39 (4). The boundaries are initially formed at a-a' of figure 39a be-

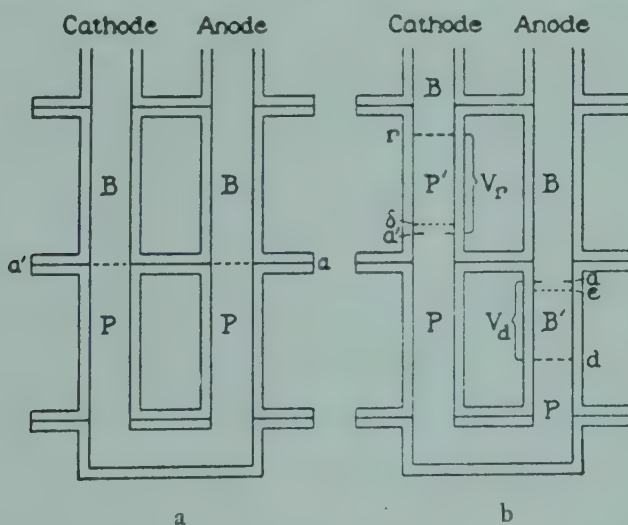


FIG. 39. Diagrams of the electrophoresis cell illustrating the formation and relative position of the boundaries. *From (4).*

tween the protein (P) and the buffer (B). On passage of current, one of the boundaries *a* migrates downward through a volume *V_d* to a new position *d*, and the other *a'* rises through a volume *V_r* to position *r* (fig. 39b).

With a solution containing *n* protein components, migrating at different speeds, *n* boundaries will be formed soon after current has been started. In the ascending limb of the U-tube the fastest moving protein will form a boundary against the buffer, the next fastest migrating protein will form one against the fastest protein, etc. Movement of the protein molecules may be followed by observing the boundaries.

The migration will be toward the anode (+) terminal if the protein is on the alkaline side of its isoelectric point and hence is negatively charged, and toward the cathode (−) pole if the protein is on the acid side of its isoelectric point and is positively charged. At the isoelectric point, no migration will take place. At any specified pH, temperature, and salt concentration, the distance (*d*) moved by a given protein boundary per unit time (*t*) will depend upon the potential gradient (*F*). For a given potential gradient, the rate of migration (*d/t*) will be characteristic for each individual protein. The potential gradient may be calculated from the conductivity of the buffer (*k*), the current (*i*) and cross sectional area of the U-tube (*q*) as follows:

$$F \text{ (volts/cm.)} = \frac{i \text{ (amperes)}}{q(\text{cm}^2) \cdot k \left(\frac{1}{\text{ohms/cm}} \right)} \quad [1]$$

The electrophoretic mobility (*u*) may be defined as the distance moved in centimeters per second under a potential gradient of 1 volt/cm. or:

$$u(\text{cm}^2/\text{volt-sec}) = \frac{d}{t F} = \frac{d q k}{i t} \left(\frac{\text{cm}}{\text{sec.volt/cm}} \right) \quad [2]$$

at pH...., in....buffer of....ionic strength.

For determinations of electrophoretic mobility, it is necessary to measure accurately the distance moved by the protein boundary, the time, the current passing and the conductivity of the solution, and to know the cross sectional area of the U-tube. The position of the

boundary at any time is determined by taking advantage of the absorption of ultraviolet light by the protein or of the difference in refractive index between the protein and the buffer.

Apparatus and Technic: In its most widely used form the Tiselius electrophoresis apparatus (1) consists of a central U-tube

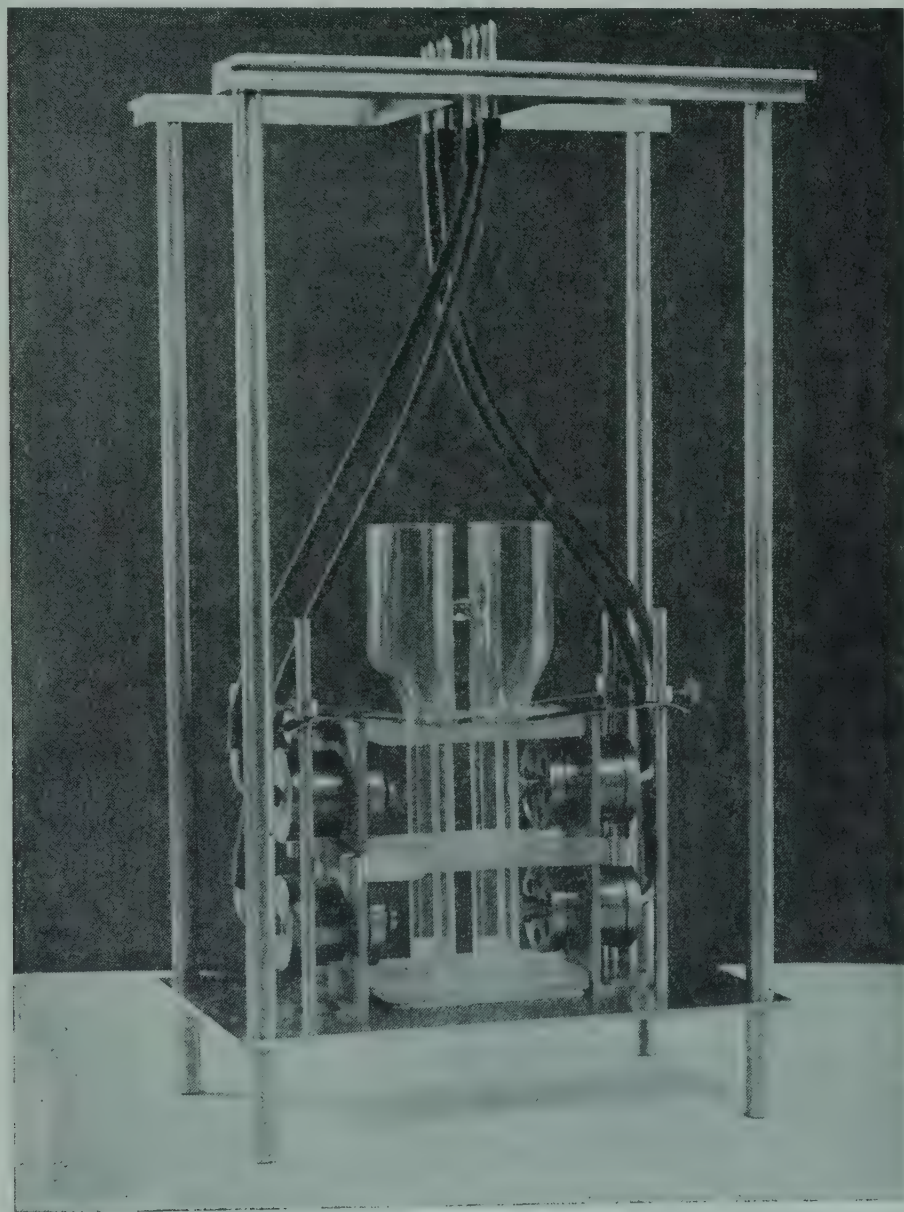
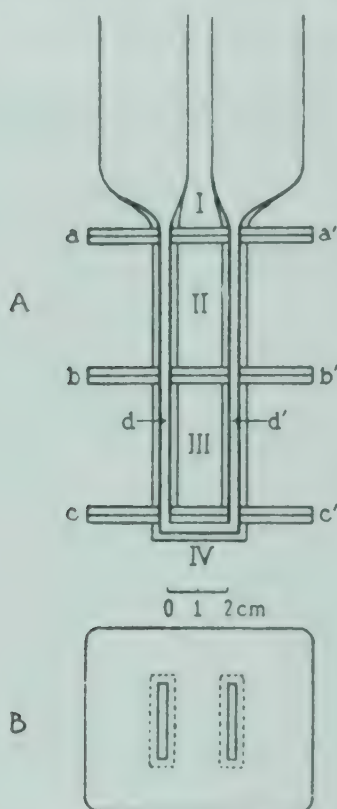


FIG. 40. The standard size Tiselius cell in its mounting. *From (5).*

constructed from glass plates cemented together to form sections. The cells are rectangular in cross section and have end walls of high

optical quality for observation of boundaries by changes in refractive index along the length of the cell. This cell consists of four sections which form the complete U-tube (fig. 41) (6). The flange



—Courtesy of Williams and Wilkins Company

FIG. 41. A, electrophoresis cells in cross section; B, top view of one of the center sections. From (6).

plates of each section are lubricated with a mixture of vaseline and paraffin oil and may be moved with respect to one another to seal off the U-tube into 5 compartments. This is advantageous for filling and emptying the apparatus and for isolation of individual components from protein mixtures. The U-tube is connected by thin-wall rubber tubing to two electrode vessels of large volume into which are placed silver-silver chloride electrodes.

The solution to be examined is dialyzed in the cold against a large volume of an appropriate buffer solution until it has the same pH and conductivity as the buffer. It is then introduced into the U-tube so that the two lower sections are completely filled. The two lower sections are then separated from the rest of the U-tube by sliding

section III out of alignment, and the protein solution remaining in section II is washed out and replaced by buffer (fig. 41). The electrode vessels are then connected and filled with buffer. A diagram of the apparatus as set up in the thermostat without the electrodes is shown in fig. 42 (5). The electrodes are introduced and

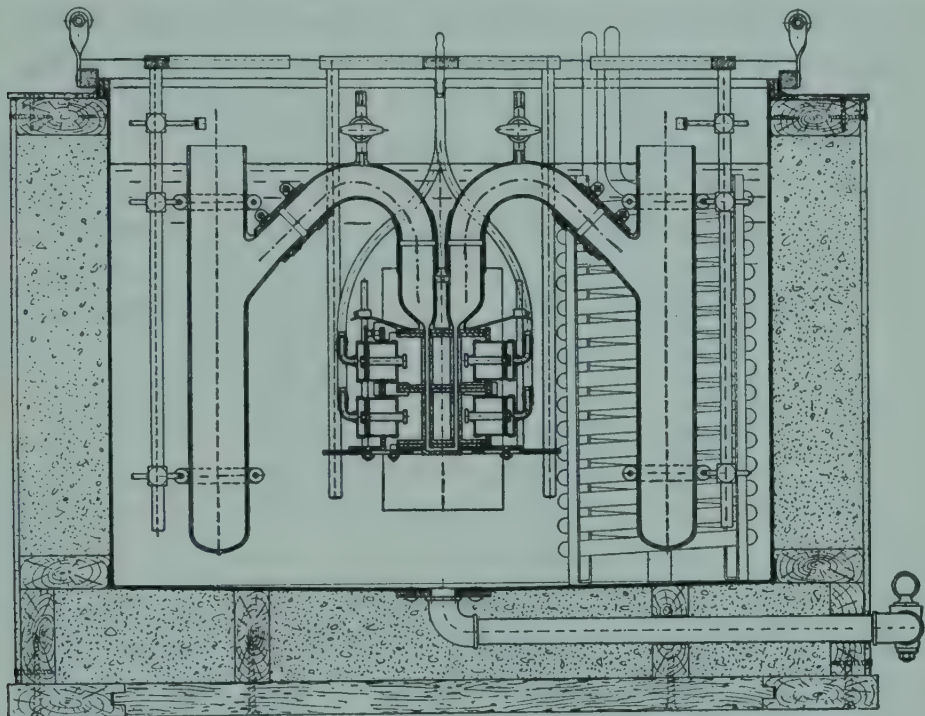


FIG. 42. Tiselius electrophoresis apparatus. *From (5).*

the entire apparatus allowed to reach temperature equilibrium in the thermostat which is usually kept at 0.5 to 1° C. The level of the liquid in the two electrode vessels is equalized by a Y-tube filled with buffer attached to the two stopcocks in the connecting arms of the electrode vessels. This prevents a difference in hydrostatic level in the electrode vessels from causing the boundary to move when the U-tube is again brought into alignment. A cold solution of saturated potassium chloride is layered under the buffer solution until the electrodes are covered. The Y-tube is then removed and section III moved into alignment so that a boundary of protein is formed under the buffer in section II. The current is then started and migration of the protein will begin. For calculating the mobility, the time of passage of the current and its intensity in milliamperes are measured and the position of the boundary is also measured, usually from photographs taken at various time intervals.

As described thus far, however, this arrangement has very limited flexibility. The initial position of the boundary is not visible since it is behind the flange plates and any slight displacement occurring when the boundary is made would introduce an error in measurement of its initial position, and therefore in the mobility. In addition, the time during which electrophoresis could be run, would be limited by the length of the cell. The resolving power of the apparatus i.e. its capacity to distinguish two components that differ but slightly in mobility would therefore not be very great and separation of one of the components of a mixture of proteins in a single compartment would become difficult. These drawbacks were overcome by Tiselius through the introduction of the so-called compensation mechanism (1). In its original form it consists of a cylindrical rod which is immersed in one of the electrode vessels when setting up the apparatus. This rod is connected to a clockwork mechanism which can raise or lower it from the solution at a slow rate. As it is raised the hydrostatic level in that electrode vessel becomes lower than the level in the other side and fluid migrates through the U-tube, thereby causing the boundary to move. The rate of compensation may be varied by the use of rods of varying size or by stopping the mechanism at intervals. This compensation mechanism is very widely used in electrophoresis to bring the boundary out from behind the flange plates so that its initial position may be observed before starting the current. It also makes possible the use of a constant hydrostatic counter current, which keeps the boundaries from migrating out of the cell so that experiments can be run for considerably longer periods thus providing increased resolving power. It is also essential for electrophoretic separations since it permits isolation of desired components in a single compartment. Compensation, however, is not used while mobility measurements are being made.

The resolution by electrophoresis of a mixture of three proteins, A, B, and C, of different mobility under ideal conditions is illustrated diagrammatically in figure 43. Fig. 43a shows the initial positions of the buffer and the protein mixture in the cell while setting up the apparatus. After equilibration in the thermostat, the displaced section is moved into alignment and the current started. Migration of each protein occurs and since protein A has the greatest mobility, it will move a greater distance than protein B which in

turn will move farther than protein C in a given time interval. Thus on the ascending side protein A will move into buffer, protein B will move through a solution of protein A and protein C through a solution of A and B. On the descending side protein A will move away most rapidly leaving a region containing B and C; protein B will move more rapidly than C, leaving a portion of solution containing only protein C. This is the condition indicated in Figure 43b.

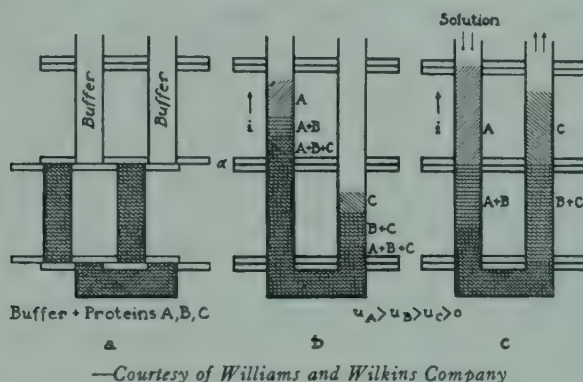


FIG. 43. Ideal electrophoresis of a protein mixture From (6).

Figure 43c shows the use of the compensating mechanism together with the current to isolate portions of the solution containing only protein A in the upper left compartment and only protein C in the upper right compartment. The current and the compensating mechanism are so adjusted that the position of the boundary of protein B moves backward until it occupies its initial position and then remains stationary. The boundary of protein C on the descending side will move slowly backward filling the upper right compartment, while that of protein A will move forward filling the upper left compartment. At the desired point both the current and compensation are stopped, the lower section (section III) moved aside and solution containing only proteins A and C respectively removed from the respective upper compartments.

Optical observation of boundaries: To measure the distance moved by a migrating boundary during a given time interval, methods for the determination of the boundary position are necessary. If the material under investigation is colored, measurements offer no difficulty. However, since most proteins are colorless, special optical methods for observing the protein boundaries had to be developed. The earliest methods (7, 3) were based on the fact that protein solutions absorb ultraviolet light of wave length about

2800 Å (cf. III, 30). By photographing the boundary using ultraviolet light its position could be determined. A microphotometer tracing of the photograph is used in determining the center of the boundary. This method had several serious drawbacks, notably that quartz cells and equipment had to be used and that only rough measurements of the concentration of the protein solution could be obtained. For details of its use see (3, 7).

More satisfactory results were obtained by the introduction of methods based on the measurement of the differences in refractive index between the buffer and the protein solution. These procedures not only make possible exact measurement of the position of the protein boundary but also permit the concentration of the protein to be determined with a high degree of precision. The two most widely used procedures are the Lamm scale method (8, 9, 7) and the Toepler "schlieren" method (1, 2). One or the other of these methods is also used almost exclusively in both ultracentrifuge and diffusion measurements.

The principle of the scale method may be readily demonstrated by holding a millimeter scale in back of a test tube containing a solution of saturated sodium chloride or of concentrated sulfuric acid carefully layered under water. At the boundary it will be observed that the scale lines are no longer uniformly spaced but that one group of lines appear to be closer together and another group farther apart. This displacement of the scale lines at the boundary is due to the changes in refractive index as one goes from one solution into the other. Fig. 44 (7) shows a photograph of a standard millimeter scale (A) and one taken through a solution containing a boundary causing a concentration gradient (B). The displacement of each scale line from the normal position is determined using a comparator (measuring microscope) and measuring the distances on each scale from some arbitrary scale line where there is no gradient. If the displacement, Z , of each scale line is plotted against the number of the scale line a curve such as that in fig. 44 results. The ordinate, Z , can be shown to represent "rate of change of refractive index" and the area under the curve is proportional to the difference in refractive index, between the buffer and the protein solution. Since the protein solution has been previously dialyzed against the buffer the difference in refractive index is due only to the protein and the area under the curve is proportional to the

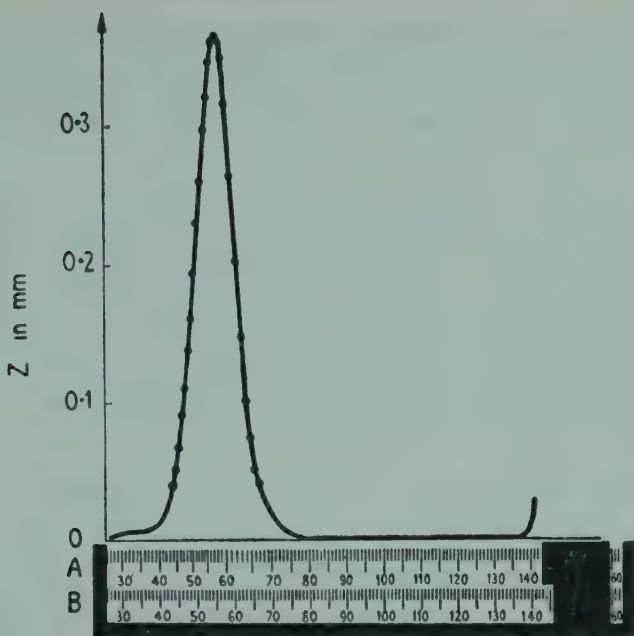


FIG. 44. Relationship between scale photographs and scale diagram. (A). Scale photograph through cell containing a concentration gradient. Note the distortion of scale A between the lines 40-70. B Normal scale photograph. *From (7).*

concentration of protein. The area under the scale diagram is measured with a planimeter. The protein concentration (c) may be calculated from the following formula (10):

$$c = \frac{A}{G \cdot a \cdot b \cdot \alpha} \cdot \frac{(l-b)}{l-G} F \quad [3]$$

where A — area under curve

G — photographic enlargement factor

a — thickness of fluid in the cell

b — optical distance from scale to middle of the cell

α — refractive index increment of solute (protein)

l — optical distance from scale to objective

F — a factor to convert the graph units to absolute units (cm)

A diagram illustrating the optics of the scale method appears in (8, 7). When the scale method is used, the camera is focused on the scale which is placed behind the cell containing the solution.

The scale method is the most accurate method of measuring concentrations. Tiselius and Horsfall (10) found the mean deviation of the concentrations determined in thirty individual scale diagrams in three separate experiments from the concentrations found by the

micro-Kjeldahl method to be ± 4.8 per cent. These measurements were made on a solution containing a single protein component. Electrophoretic patterns obtained with the scale method are shown in (IV, 43 Fig. 87).

Measurement of scales in a comparator is very tedious and time consuming. The labor involved with the scale method has been eliminated by the introduction of two technics for direct photographic recording of refractive index changes throughout the cell (4, 11-13). These procedures are based on the Toepler "schlieren" method and permit direct observation of the boundaries on a ground glass screen.

One of the methods, the Longworth schlieren-scanning method (4) is illustrated in fig. 45:

"An image at P of the illuminated slit S-S is formed by the schlieren lens D. The camera lens O is focused on the electrophoresis cell E and forms an image (full sized in most types of apparatus) on the screen at G-G. If the fluid in the cell is homogeneous this image will be uniformly illuminated. On the other hand, if there is a boundary, B, between, for instance, a protein-bearing solution and a buffer, there will be a region in which the refractive index varies with the height in the cell, and light which would normally pass to P is deflected downward, since the solution with the greater refractive index is on the bottom. If the opaque schlieren diaphragm Q is raised to a point p where it intercepts the most deflected light, a dark band will appear on the screen, conjugate to the region of steepest gradient in the boundary B. Such a band appears in the image G-G at p'.

"However, a boundary between two solutions does not consist of a single geometric plane, but of a region in which the composition

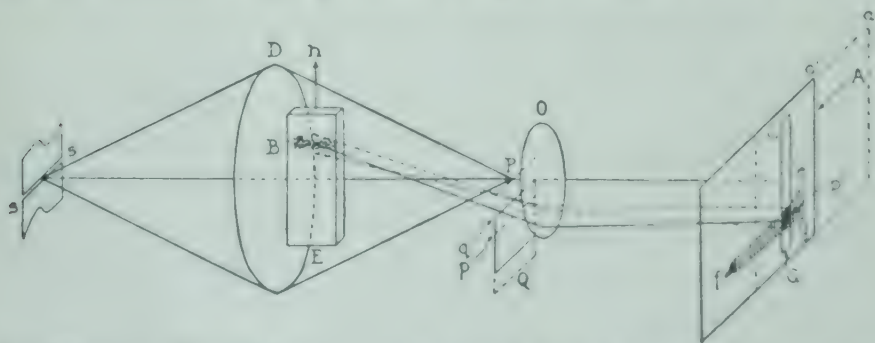


FIG. 45. Diagram of the schlieren scanning method for the photographic recording of gradients of refractive index. From (11).

varies from that of one solution to that of the other. The refractive index, n , in such a region changes continuously with the height, h , of the liquid in the cell. The gradient, dn/dh , of refractive index, for each boundary, will thus, theoretically at least, vary from zero to a maximum, and back to zero. In fig. 45 the variation of the gradient, dn/dh , of the boundary B in the cell E, is represented by the density of the shading. The pencil of light passing through the layer having the maximum value of the gradient will be most bent from the normal path and will be the first to be intercepted when the schlieren diaphragm Q is raised. On lifting this diaphragm still further, i.e., to the level q, less refracted pencils of light will also be intercepted and the schlieren band in the image G-G will widen. This obviously can be continued until the whole field has been covered. In the schlieren scanning method this process is made continuous and is recorded photographically. The image of the cell at G-G is masked by a narrow vertical slit and a photographic plate A is moved in the direction of the arrow at a constant rate across this slit. Actuated by the same mechanism, the schlieren diaphragm Q is given a steady movement upward. The resulting (positive) photographic record for a typical single boundary is indicated by the area e-f-g. The displacement of the diaphragm Q from the position P is proportional to the gradient at levels in the cell E conjugate to the edges of the schlieren bands. Thus the contour of the area e-f-g indicates both the position and the magnitude, of the refractive index gradients existing in the boundary. Since the photographic plate A was in position a at the time the schlieren diaphragm was at p, a section of the band p' appears at f when the plate has been moved to a'. It will be seen that the usual schlieren bands are narrow sections through the area e-f-g. By a system of gears the ratio of the rates of motion of the schlieren diaphragm Q to that of the plate A is given a constant value, such as one to three, and this ratio can be varied by changing the gears. For establishing the base line of the schlieren patterns the position of the diaphragm Q may be read accurately with a micrometer."

The second of these methods developed by Philpot (13) and Svensson (11) is known as the cylindrical lens method. It is illustrated in fig. 46 by Longworth and has been described (12) as follows: "An illuminated horizontal slit, present on the left of S but not shown in the figure, is focused by means of the schlieren

lens, *S*, in the plane of the schlieren diaphragm, *D*. The latter contains a diagonal slit, *kk*, as shown in the front view, *D'*. The camera objective *C*, is focused on the electrophoresis cell, *E*, and forms, in the absence of the lens *H*, a normal image of the cell on the ground-glass or photographic plate at *G*. The cylindrical lens, *H*, with its axis vertical, is focused on the schlieren diaphragm and also on the plate at *G*. Viewed from the side (figure 46a), the cylindrical lens has no effect on the pencils of light forming the cell image. Thus the vertical coordinate of each point in the image is conjugate to the corresponding level in the channel *E* and, owing

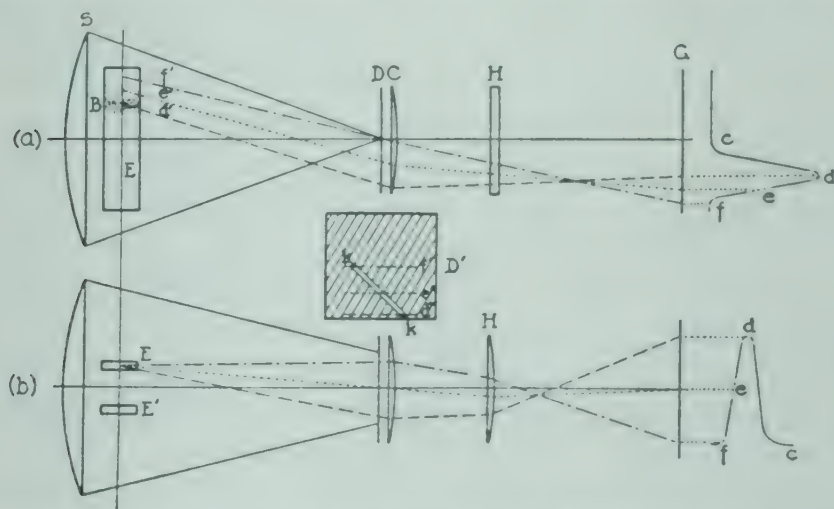


FIG. 46. Diagram of the cylindrical lens method for the observation of the electrophoretic patterns. From (13).

to the focusing action of the camera lens, *C*, this also remains true for pencils that may be deflected by gradients in the channel. Viewed from above, however (figure 46b), the cylindrical lens, in conjunction with the diagonal slit, causes, as will be shown below, a lateral deviation of a pencil of light that is proportional to the vertical deflection the pencil has suffered in a boundary. The curve to the right of *G* in figure 46a represents the pattern of the boundary, *B*, as it would appear on the screen if the latter were hinged at the side and turned toward the reader, whereas if it were hinged at the top and turned, the pattern would appear as in figure 46b.

"If the fluid in the electrophoresis cell is homogeneous, all of the light through the channel is concentrated in an image of the illuminated slit at the upper or normal level on the diaphragm, i.e., *f'* of *D'*, or *D*. As can be seen from the figure, only the extreme left-

hand portion of the light in this image passes through the diaphragm to form a straight vertical line, i.e., with base line, on the screen at the position c-f. The width of this line varies with the width of the diagonal slit, and since a wide line is undesirable, Svensson has made the practical suggestion of tapering the end of the slit *kk* to a point.

"If, on the other hand, a boundary *B* is present in the cell, a pencil through the layer of solution in the boundary having the maximum gradient, for example, is deflected downward as indicated by the line *d'd* and forms an image of the slit at the lower level *d'* on the diaphragm. Owing to the angle the diaphragm slit makes with the vertical, the portion of the light in the lower image *d'* that enters the slit is shifted laterally from the position at which the normal pencil enters by an amount proportional to the vertical deflection in the boundary gradient. The cylindrical lens consequently imparts to this pencil a corresponding lateral shift in the opposite direction to the position *d*, figure 46b, without affecting its vertical position, i. e., *d* of figure 46a.

"The path of a pencil through another portion of the boundary is indicated by the line *e'-e* and forms a corresponding element in the pattern. All other elements in the complete pattern are formed similarly."

Specific Refractive Index Increment: The constant, α , is defined as the difference in refractive index (*N*) of a solution containing 1 gm. of protein per 100 ml. of solution and the refractive index of the solvent or buffer for light of a specified wave length. It may be calculated by the following formula:

$$\alpha = \frac{N \text{ solution} - N \text{ solvent}}{\text{protein concn. (gm/100 ml)}} \quad [4]$$

Values for the refractive index increment of crystalline horse serum albumin of 0.00183 and of horse serum globulin of 0.00186 were obtained by Adair and Robinson (14) for light of wave length of the D line of sodium. Albumin and globulin from sheep serum were found to have specific refractive index increments of 0.00185. Accurate measurements of the refractive index increment have still to be made on some of the electrophoretic components of serum, notably the alpha and beta globulins. In the absence of the measurements relative concentrations of these components have been cal-

culated from the area measurements without including the refractive index increment.

Electrophoretic Patterns of Proteins: Electrophoretic measurements have been widely used in characterizing purified protein preparations. The electrophoretic mobility measured in a specific buffer at a given pH and ionic strength is a reproducible physical constant. By carrying out measurements at different pH the isoelectric point may be calculated by plotting a graph of mobility against pH and locating the pH at which the mobility is zero.

If refractive index methods are used, the presence of even relatively small amounts of other proteins may be detected. If their mobility differs sufficiently from the main component two peaks will be observed and the concentration of the impurity may be calculated. Frequently two components may have the same mobility at one pH and yet can be resolved at another pH. As a test of electrophoretic homogeneity, measurements should be carried out over as wide a pH range as the stability of the protein will permit.

One of the most important uses of electrophoresis has been in the examination of naturally occurring mixtures of proteins, notably serum or plasma. The number of components found in serum or plasma depends upon the pH and the buffer used. In immunochemical studies a buffer containing 0.15 *M* NaCl + 0.02 *M* phosphate (9) buffered at the desired pH from 6 to 8 has been widely used, so that quantitative precipitin analyses could be carried out on separated fractions directly without the necessity of dialyzing samples against saline. In this buffer at pH 7-8 normal serum from various animal species shows 4 protein components. The fastest component is albumin and the others have been designated in order, alpha, beta and gamma globulins. (Other so-called delta and epsilon boundaries are not protein components and will be discussed below). The most satisfactory electrophoretic patterns have been obtained with serum diluted 1:4. Essentially similar patterns have been obtained in 0.1 ionic strength phosphate buffer (15) or in a pH 7.8 veronal buffer consisting of 0.025 *M* lithium diethyl barbiturate, 0.025 *M* diethyl barbituric acid, and 0.025 *M* LiCl (16), but these are not to be recommended if direct quantitative immunochemical methods are to be used on the same samples unless the effect of the buffer on the immune system is first studied. When plasma is examined an additional boundary is observed between the beta and

gamma globulin and is due to fibrinogen (ϕ) (16, 17). Electrophoretic patterns of normal human serum and plasma in barbiturate buffer at pH 7.8 are shown in fig. 47 (16); the sharp line is the so-

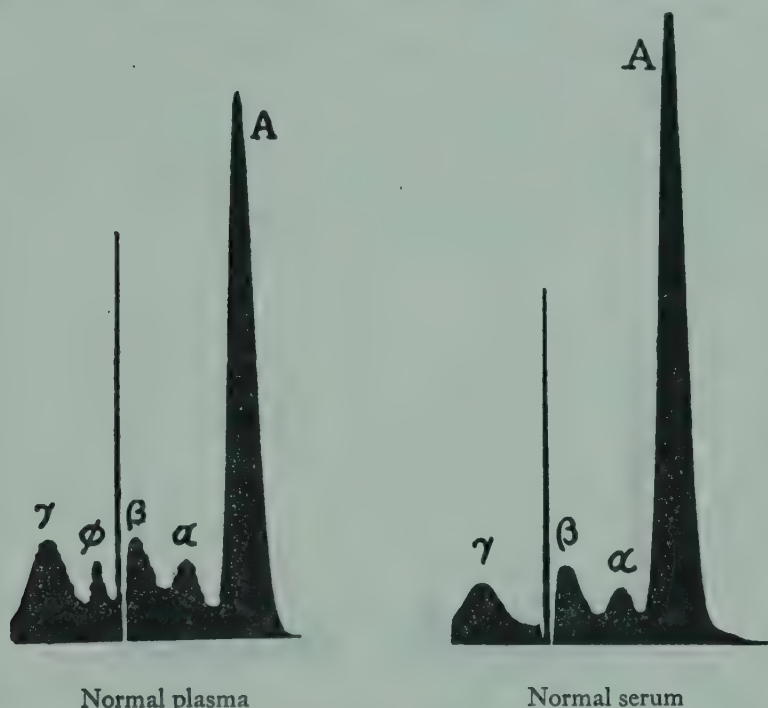


FIG. 47. Electrophoretic patterns of normal human serum and plasma diluted 1:4 at pH 7.8 in a buffer consisting of 0.025M lithium diethyl barbiturate, 0.025M diethyl barbituric acid and 0.025M LiCl, ionic strength 0.1. *From (16).*

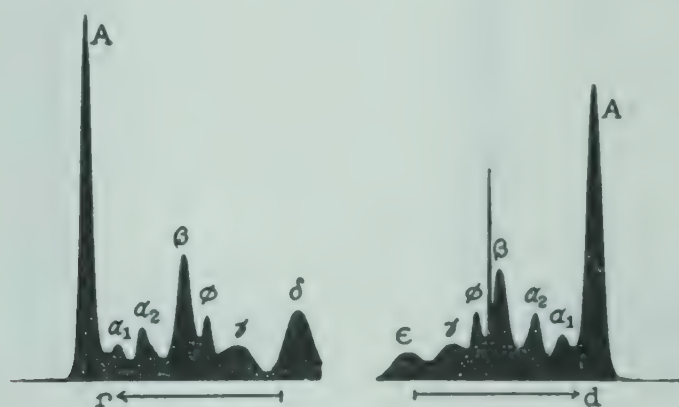
called beta anomaly; for the patterns of an immune rabbit serum before and after removal of antibody (see IV, 43, fig. 87).

If a pH 8.6 buffer containing 0.1 *M* sodium diethyl barbiturate (V) and 0.02 *M* diethyl barbituric acid is used, greater resolution is obtained and a new component called α_1 is separated from the albumin component. The ascending and descending patterns of normal human plasma obtained under these conditions are shown in fig. 48 (12).

The mobility of each of the components present may be calculated from the distance which it moved over a given time interval as outlined above. From a consideration of boundary anomalies (see below), mobility values for the descending side are more reliable. In studies on a number of sera or plasmas, variations in mobility of ± 10 per cent for each component are not uncommon (16).

At the present time the absolute concentrations of each com-

ponent in plasma can not be calculated since the refractive index increments of all the various protein components are not known. However, concentrations in terms of refractive index change (i.e., area) can be calculated as described for the scale method, but omitting the specific refractive index increment term. Such relative concentrations are widely used, and if the percentage composition of the components of a mixture as measured electrophoretically and the total nitrogen content of the solution examined are specified, results of different laboratories may be compared.



—Courtesy of Williams and Wilkins Company

FIG. 48. Electrophoretic patterns of plasma diluted 1:2 and dissolved in a 0.1 N NaV-0.02 N HV buffer at pH 8.6. Patterns were obtained after electrolysis for 14,000 sec. at 5.38 volts per centimeter. *From (12).*

Measurements of relative concentration are carried out from an enlarged tracing of the electrophoretic pattern. The procedure described by Tiselius and Kabat (9) is the simplest and most widely used. It arbitrarily separates the components by drawing an ordinate at the lowest point between two adjacent peaks as shown in fig. 49 (16) and measures the area of each component with a planimeter. The area of any component divided by the total area represents the percentage of that component in solution in terms of refractive index. The method cannot be used with precision unless adequate separation between components is obtained, and the lowest point between peaks is fairly close to the base line. Longworth (12) has used an alternate method of separation of the components by resolving the pattern into a series of symmetrical curves as is usually done for ultracentrifugal patterns (7), but agrees that the former method is simpler. As the separation of two components becomes

more complete, the values obtained by both methods give better agreement.

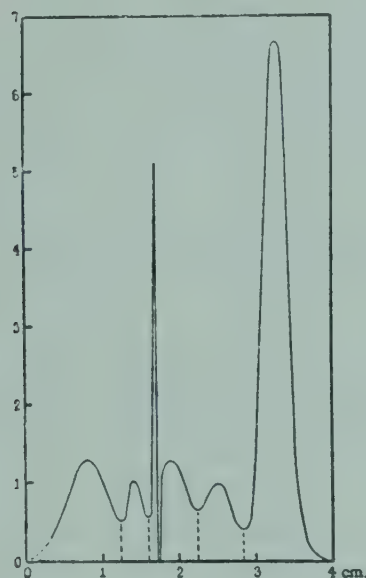


FIG. 49. Tracing of electrophoretic pattern for estimation of concentrations. *From (16).*

Electrophoretic analysis has been widely used as a guide to chemical fractionation of protein mixtures such as plasma or serum (18-21), cerebrospinal fluid (22), the proteins of egg white (23), bacterial proteins (24, 24a), etc. It has also been of value in studies on variations in plasma proteins accompanying disease (16, 25, 26) and of compound formation between nucleic acid and proteins (27). Electrophoretic studies on antibodies (28) are included in (I, 6 and IV, 43).

Boundary Anomalies in Electrophoresis: Under actual conditions of electrophoresis certain boundary anomalies appear. These have been treated by several workers (1-5, 12, 29) and must be considered in the interpretation of data. Svensson (29) points out that ideal electrophoresis takes place when the mobilities of all ions and the conductivity of the solution are constant throughout the electrophoresis tube and are independent of time. When these conditions do not prevail boundary anomalies occur as follows:

1. Ascending boundaries (protein into buffer) migrate more rapidly and sweep out larger volumes than descending boundaries.
2. Ascending boundaries are sharper than descending boundaries.
3. At the starting position two false boundaries appear, called the

delta and epsilon boundaries (fig. 48) on the ascending and descending sides respectively. The delta boundary is more pronounced than the epsilon boundary.

4. On the ascending side there is a gain of electrolyte and on the descending side a loss of electrolyte, i.e., the conductivity, if initially constant, decreases on the ascending and increases on the descending side.

Svensson also gives a mathematical development of electrophoretic theory with respect to boundary anomalies. (cf. 34, 35).

These boundary anomalies are very important in any consideration of electrophoretic data, since they affect both mobility and concentration measurements. Thus, certain buffers, notably the pH 7.8 barbiturate buffer do not effect the separation of the delta and epsilon boundaries from the gamma boundary, thereby introducing an error in the measurement of the gamma globulin area. Other buffers such as phosphate buffers and the pH 8.6 barbiturate buffer permit separation of the gamma component from the delta and epsilon boundaries. With this latter buffer mobilities and concentrations calculated from ascending and descending boundaries were found to be identical (29a).

Of considerable importance is the finding of Svensson (29) that addition of NaCl to the buffer depresses the boundary anomalies. Svensson examined normal serum containing 6 mg. N per ml. in $0.032M$ Na_2HPO_4 + $0.004M$ NaH_2PO_4 containing varying amounts of NaCl from 0.00 - $0.37M$. With increasing salt concentration the delta boundary decreased and the percentage of albumin as measured electrophoretically also decreased. Mobilities and concentrations as measured from ascending and descending boundaries approached one another. With serum diluted with an equal volume of buffer, $0.3M$ NaCl was sufficient to reduce the boundary anomaly errors to the magnitude of accidental errors. For a 1:4 dilution of serum $0.15M$ NaCl would suffice. This is fortunate since these conditions are optimal for immunochemical studies. These studies should also serve to emphasize the limitations in comparing the data obtained under varying conditions. A buffer containing $0.15M$ NaCl + $0.02M$ phosphate would be suitable for solutions containing up to about 1.5 per cent protein. A recent note suggests, that these considerations do not apply to barbiturate buffer at pH 8.6 (29a).

Limitations of Electrophoretic Analysis: There are numerous pitfalls in electrophoretic analysis which arise chiefly from inadequate understanding of the limitations of the technic and from failure to consider these limitations in quantitative terms. For example, if a purified protein is examined electrophoretically and found to be homogeneous, how much of a second component could still be present and yet not be detected? The answer to this question depends upon several factors. If the mobility of both components is the same at the pH of the experiment, a mixture of the two in any proportions will not be resolved. If there is a difference in mobility of several units, however, the presence of a small proportion of a second component may readily become apparent. Under ordinary conditions a solution containing 0.02-0.03 per cent protein can just be resolved electrophoretically. Thus if a 1% solution of the protein is examined, 2-3% of a component with a different mobility could be detected; by the use of a 3% solution, as little as 1% of impurity could be detected. Because of boundary spreading a component present in such small proportions will be visible for only short periods during the run. It will also be more readily detectable if it migrates more rapidly than the main component, since more slowly migrating components may be confused with the delta or epsilon boundary.

It is apparent, therefore, that in a complex mixture of proteins such as plasma or serum where components having a wide range of mobility are present, much greater variations must occur to be detectable by gross examination of the electrophoretic diagrams. It is doubtful whether an additional component constituting even as much as 5 per cent of the total protein present could be resolved as a separate peak.

Greater sensitivity in detecting variations in the patterns of protein mixtures may be obtained from area measurements under each peak as described above. However, since the delineation of the area under each peak is somewhat arbitrary, differences in area of several per cent may not be significant.

It follows that if a protein constitutes less than 1 or 2 per cent of the total protein present as in the case of complement, enzymes, or antibodies formed during convalescence, its detection from electrophoretic patterns is impossible.

Using hyper-immune rabbit and monkey sera in which the anti-

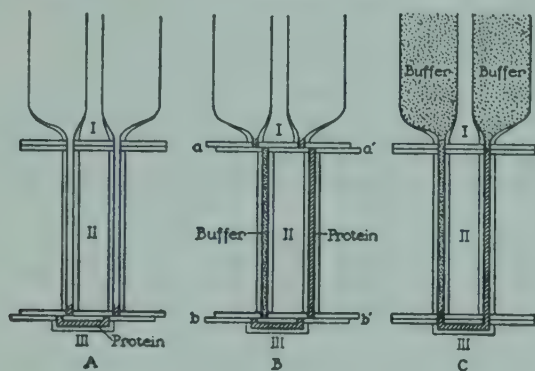
body comprised 6 to 36 per cent of the total protein as determined by the quantitative precipitin method, Tiselius and Kabat (9) (see IV, 43) were able to show the exclusive association of the antibody with the gamma globulin fraction by measuring the decrease in area of the gamma globulin peak after removal of the antibody by addition of antigen. This method was pushed beyond its limit of sensitivity, however, in an electrophoretic examination of rabbit antisera to one of the proteins of tuberculin (30) and the conclusion was drawn that this antibody was associated with the alpha globulin or the albumin fraction. Critical appraisal of the data shows that the antisera employed were so weak that removal of the antibody would cause no significant alteration in the electrophoretic pattern. Furthermore, a decrease in area of alpha globulin after removal of antibody was accompanied in every instance by an almost corresponding increase in the albumin or beta globulin area and vice versa, suggesting that the differences were caused by slight variations in the point at which the ordinates separating the albumin from the alpha globulin and the alpha from the beta globulin were drawn.

When the substance under investigation constitutes only a small proportion of the total protein but has some unique biological activity (enzymes, hormones, antibodies, etc.), some data may be obtained by separating various components electrophoretically and determining with which of these components the activity migrates. If the substance moves together with a definite component such as the gamma globulin, for example, serum fractions containing alpha and beta globulins will be inactive while the gamma globulin will show activity. In this manner Newell (31) has established that the antibody in the serum of ragweed-sensitive patients is associated with the gamma globulin fraction. In some instances, the mobility of the active substance does not correspond to any of the visible components. Under such circumstances, a number of separating runs must be made to determine between which components the activity migrates. This has been done with human Wassermann antibody (32) which was found in this way to have a mobility intermediate between that of the beta and gamma globulins.

MISCELLANEOUS NOTES

1. A modified Tiselius cell in which a double length section is

substituted for section II and III of figure 4I is shown in figure 50. The method of filling the cell is apparent.



—Courtesy of Williams and Wilkins Company

FIG. 50. Diagrams illustrating the initial formation of the boundaries in the Tiselius electroresis cell with the tall center section. From (12).

2. With solutions too opalescent for obtaining good photographs by visible light, infra-red photography has proven of value (33).

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CHAPTER 26

ULTRACENTRIFUGAL ANALYSIS

During the past two decades the pioneering researches of Svedberg, Pedersen and their collaborators have made available to the protein chemist one of the most valuable tools for the characterization of proteins. By its use not only have the molecular weights of proteins been precisely determined, but a means of analyzing both naturally occurring and artificial mixtures of proteins of different molecular sizes has been made available. The ultracentrifuge together with the Tiselius electrophoresis apparatus has become indispensable for study of high molecular weight substances and the progress of immunochemistry has been materially advanced by their development. The purpose of this section is not to enable the immunochemist to construct or operate the instrument but rather to understand the fundamental principles and to analyze ultracentrifugal data critically and use ultracentrifugation as a research tool. For further information and details, the book of Svedberg and Pedersen (1) is highly recommended.

Two ultracentrifugal methods have been developed for determination of molecular weights; the sedimentation equilibrium and the sedimentation velocity methods. The former is based upon measurements of the concentration gradient developed when a protein solution at constant temperature is exposed to an external centrifugal (or gravitational) field of such intensity that equilibrium between the centrifugal and diffusion forces is established. This method has proven of limited utility in the study of proteins except for the determination of molecular weights since it provides little information about the homogeneity or heterogeneity of the solution under investigation and will not be considered further; details may be found in (1).

The sedimentation velocity method measures the sedimentation of a protein when subjected to a centrifugal field of known intensity for a given time. As sedimentation proceeds the position of the boundary between the protein and buffer is measured at various time intervals. From these measurements a constant, s , the sedi-

mentation constant may be calculated. The sedimentation constant is defined as follows:

$$s = \frac{dx/dt}{\omega^2 x} \quad [1]$$

where dx/dt = the rate of sedimentation in cm. per sec., i.e., the distance moved by the boundary per unit time.

x = the distance from the center of rotation to the protein boundary in cm.

ω = the angular velocity in radians per sec.

On integration equation [1] becomes

$$s = \frac{\ln x_2/x_1}{\omega^2(t_2-t_1)}$$

which becomes approximately

$$s = \frac{2(x_2 - x_1)}{(x_2 + x_1) \omega^2 (t_2 - t_1)} \quad \frac{\text{cm}}{\text{sec} \cdot \text{unit field}} \quad [2]$$

where x_2 and x_1 are the distances from the center of rotation to the boundary at times t_2 and t_1 . This is the form of the equation most frequently used for calculation of the sedimentation constant. If x_2/x_1 is < 1.4 , the error introduced by the approximation is less than one per cent.

The sedimentation constant, as obtained above, varies with the temperature at which the measurements are made and with the density and viscosity of the solvent (including salts) in which sedimentation occurs. For comparison of results obtained under varying conditions, it is necessary to reduce sedimentation constants to an arbitrary set of standard conditions, generally to water at 20° C. The sedimentation constants obtained from equation [2] are corrected to standard conditions by the following formula (1):

$$S_{20}^{\circ} = S_t \cdot \frac{\eta_{20}^{\circ}}{\eta_t^{\circ}} \cdot \frac{\eta_t}{\eta_{20}} \cdot \frac{1 - V_{20}\rho_{20}^{\circ}}{1 - V_t\rho_t} \quad [3]$$

S_{20}° = the sedimentation constant at standard conditions

S_t = the sedimentation constant obtained from equation [2]

$\eta_t^\circ / \eta_{20}^\circ$ = ratio of the viscosity of the H_2O (or of solvent if non-aqueous media are used) at temperature t to its viscosity at 20°

η_t / η_t° = ratio of the viscosity of the buffer solution in which the measurements are carried out at temperature t to the viscosity of water at the same temperature

V_t = partial specific volume of protein at $t^\circ \text{C}$.

V_{20} = the partial specific volume of the protein at 20°C .

ρ_{20}° = density of water at 20°C .

ρ_t = density of medium at $t^\circ \text{C}$.

Sedimentation constants are expressed in Svedbergs where one Svedberg (S) = 10^{-13} times the absolute unit (3).

In computing the sedimentation constant from data, the value of $S_t \cdot \eta_t^\circ / \eta_{20}^\circ$ is calculated for all of the small time intervals between successive exposures since the variation of viscosity with temperature is large. The temperature of the solution increases at a rate depending upon the velocity and time of centrifugation and must be measured during these intervals, so that the correction may be applied with precision. The other correction factors vary but slightly with temperature and are calculated for the average temperature and applied to the average of $S_t \cdot \eta_t^\circ / \eta_{20}^\circ$ obtained for the entire run to yield S_{20}° . For most proteins, $(1 - V_{20} \rho_{20}^\circ)$, may be taken as 0.2523.

The corrected sedimentation constant is used for the calculation of molecular weight together with the diffusion constant (III, 27) and in some instances with viscosity measurements (see III, 28). From the sedimentation and diffusion constants, the molecular weight may be calculated according to the following formula (1):

$$M = \frac{RT S_{20}^\circ}{D_{20}^\circ (1 - V_{20} \rho_{20}^\circ)} \quad [4]$$

where M = molecular weight R = the gas constant
 (8.313×10^7)
 T = absolute temperature
 S_{20}° = sedimentation constant in absolute units
 D_{20}° = diffusion constant

V = partial specific volume of the solute (protein)
 ρ_{20} = density of the solution at 20° C.

Equation [4] holds for dilute solutions, i.e., under conditions in which the gas laws are valid. The molecular weight obtained is, however, independent of the shape of the particle (1). For calculation of molecular weights, separate sedimentation and diffusion measurements are usually carried out, but both values must be referred to the same solution at the same protein concentration. Values are usually determined at low protein concentrations. Sedimentation constants may be extrapolated to zero protein concentration.

From the sedimentation constant and molecular weight or from the diffusion constant in dilute solutions, it is possible to obtain some information about the shape of the molecule by calculation of the frictional ratio f/f_0 . The molar frictional constant, f , may be calculated from the equation:

$$f = \frac{M(1 - V\rho)}{s} \quad [5]$$

and is a measure of the frictional force opposing sedimentation. If conditions are such that the gas laws are valid, f may be calculated from diffusion measurements

$$f = \frac{RT}{D} \quad [6]$$

and it is obvious that equation [4] may be obtained by equating [5] and [6]. These equations hold only for non-oriented sedimentation, which due to strong Brownian movement usually takes place in protein solutions during centrifugation (1).

The molar frictional constant, f_0 (1), for a compact spherical and unhydrated particle of the same molecular weight, may be calculated as follows:

$$f_0 = 6\pi\eta N \left(\frac{3MV}{4\pi N} \right)^{1/3} \quad [7]$$

The frictional ratio, f/f_0 , is obtained from [6] and [7]. If this ratio, is 1.0 for any substance, it must consist of compact, spherical, and not appreciably hydrated molecules. If f/f_0 is greater than 1, the

molecules must be either hydrated or non-spherical, or possibly both hydrated and aspherical.

From measurements of sedimentation and diffusion constants, f/f_0 may be calculated directly as shown in (1):

$$f/f_0 = 10^{-8} \left(\frac{1 - V\rho}{D_{20}^2 \cdot s_{20}V} \right)^{1/3} \quad [8]$$

This relation is obtained from equations [4], [6] and [7].

Svedberg and Pedersen (1) give tables of the ratio of length to width corresponding to varying values of f/f_0 assuming oblong or oblate ellipsoids, and unhydrated molecules.

When electrically charged particles, such as proteins, are sedimented, abnormal sedimentation velocities may be found. Two types of charge effects are responsible, designated as primary and secondary. The primary effect results from the difference in sedimentation constant between the high molecular weight protein ion and its low molecular oppositely charged partner. The effect is greatest when no other ions are present and is generally reduced by the addition of neutral salt. For a 1 percent protein solution, 0.2 *M* NaCl or KCl will be adequate to reduce the magnitude of the charge effect below that of the accidental errors. The secondary charge effect is usually small and is due to the presence of substances other than the protein, whose positive and negative ions have different sedimentation constants; it is eliminated by the addition of salts having equally dense anions and cations (NaCl, KCl) (1). The primary charge effect always reduces the sedimentation rate, whereas the secondary charge effect may either increase or decrease the observed sedimentation constant.

If the molecules of a substance are markedly aspherical and have a high intrinsic viscosity (cf. III, 28), the sedimentation constant is found to depend upon the concentration of the protein solution examined. Horse antipneumococcal antibody (see I, 6) and tobacco mosaic virus (2) were found to show such behavior. For calculating molecular weights from equation [4], the true value of the sedimentation constant has been obtained by extrapolation to zero protein concentration. Recently Lauffer (2) reported that if the observed sedimentation constant is corrected by substituting the viscosity of the protein solution for the viscosity of the solvent (liquid plus salts) in equation [3], values independent of protein

concentration and in agreement with that obtained by extrapolation to zero protein concentration are obtained. This relationship was found to hold not only for tobacco mosaic virus but also for other proteins and polymers for which both ultracentrifugal and viscosity data were available. It does not as yet appear to have any clear theoretical basis, but should prove extremely useful as an empirical rule.

Partial Specific Volume (V): To calculate molecular weights from equation [4], the partial specific volume of the protein must be determined (1, 3). The partial specific volume is defined as the change in volume produced by adding one gram of solute to an infinite amount of solvent. For dilute protein solutions, the partial specific volume is independent of concentration and the apparent partial specific volume may be used. This may be obtained by measuring the density of a protein solution of known concentration. The density is usually measured with a pycnometer and the protein concentration determined by drying a weighed amount of solution to constant weight over phosphorus pentoxide at 115° C. From these data the apparent partial specific volume may be calculated from the following formula:

$$V_{\text{apparent}} = \frac{m_0 - (m - h)}{\rho_0 h} \quad [9]$$

where m_0 = weight of pycnometer contents when filled with solvent
 m = weight of pycnometer contents when filled with solution
 h = $pm/100$ gu; p = concentration of solution as per cent by weight
 g = concentration of solution in grams per ml.
 u = volume of pycnometer
 ρ_0 = density of the solvent

When measurements are made on proteins dissolved in dilute salt solutions, the salt solution is considered to be the solvent.

The partial specific volume of most proteins has been found to be about 0.75, but several proteins have considerably lower partial specific volumes; the partial specific volume of ribonuclease is 0.71,

of horse antipneumococcal antibody 0.715, and calf thymus nucleohistone 0.66.

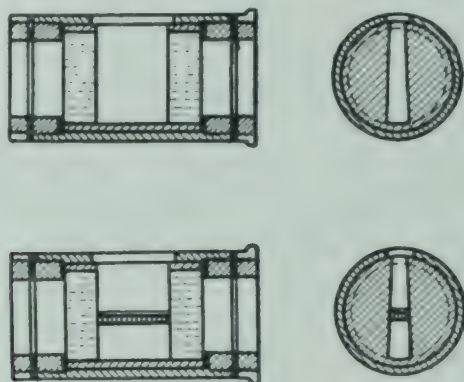
An alternate method proposed by McBain (4) measures the reciprocal of the density of the solution in which no sedimentation occurs. The method, however, has several disadvantages since the substance added to increase the density may react with or alter the protein and since the method cannot be applied to proteins with low sedimentation constants (3). In addition, if the protein is hydrated, the value obtained corresponds to the specific volume of the hydrated form. If a satisfactory inert substance for increasing the density of the medium could be obtained, a comparison of the partial specific volumes obtained by the two methods would provide information about the degree of hydration of the protein. Adair and Adair (5) have estimated the degree of hydration of protein crystals from differences in the densities of several proteins as crystals and in solution (3).

Apparatus: Several types of ultracentrifuges are in use, the oil turbine centrifuge (1), an air driven type (1, 1a) and an electrically driven, magnetically supported model (6). Rotors have also been designed to permit concentration of high molecular weight substances by ultracentrifugation. These rotors have a capacity of from 60 to 100 ml.

The Svedberg oil-turbine ultracentrifuge has been used to obtain centrifugal fields from 15,000 to 750,000 g. (cf. appendix), the limit at which the rotor will run without exploding. Rotors are constructed of a special nickel-steel alloy. The rotor is supported in horizontal bearings, is driven by twin oil turbines, and rotates in an atmosphere of hydrogen gas under reduced pressure (about 20 mm.) to diminish friction. Two holes are cut in the rotor to accommodate the cell for the solution and a cell for balancing. The usual distance from the center of rotation to the middle of the cell (R) is about 6.5 cm. for fields up to 300,000 g.

The cells used are sector-shaped to avoid convection and are provided with plane parallel quartz windows to permit optical observation of sedimentation. The height of the column of solution is usually about 13-19 mm. Care must be taken to have the cell aligned correctly within the rotor to prevent sedimentation of particles against the sidewalls (7). Cells permit varying thicknesses of solution from 1.5 to 12 mm. to be examined. A counter-

poise is placed in the opposite hole of the rotor. This balance cell permits light to pass through it and provides a reference point for the distance from the center of rotation. Front and side view diagrams of the standard cell and of a cell containing a central perforated membrane to permit separations of protein components under optical control are shown in figure 51 (8).



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FIG. 51. SECTION OF CELLS

Upper. Cell for optical observations

Lower. Cell for separation under optical control. *From (14).*

A diagram of the oil-turbine centrifuge installation is shown in figure 52 (1).

Photographs of the migrating protein boundary are taken at several time intervals using light absorption, the scale method, the Longworth schlieren scanning or the Philpot-Svensson cylindrical lens methods. These have been described in detail in the chapter on electrophoretic analysis (III, 25). From these photographs the position of the boundary and the number and concentration of the protein components is determined (III, 25).

The air driven vacuum type ultracentrifuges are considerably less costly. They employ compressed air for supporting and driving a cone shaped turbine from which the rotor is suspended by a straight piano wire as a drive shaft. The duralumin rotor (fig. 53) (1a) is enclosed in a steel casing which is evacuated to reduce friction and avoid heating. A diagram of the apparatus is shown in figure 54. The optical system is similar to that used in the oil turbine ultracentrifuge. Recently Rothen (3) has introduced a cooling coil connected to a Frigidaire unit and has substituted hydrogen at 0.1 mm. Hg. for the vacuum. This permits very close regulation of the

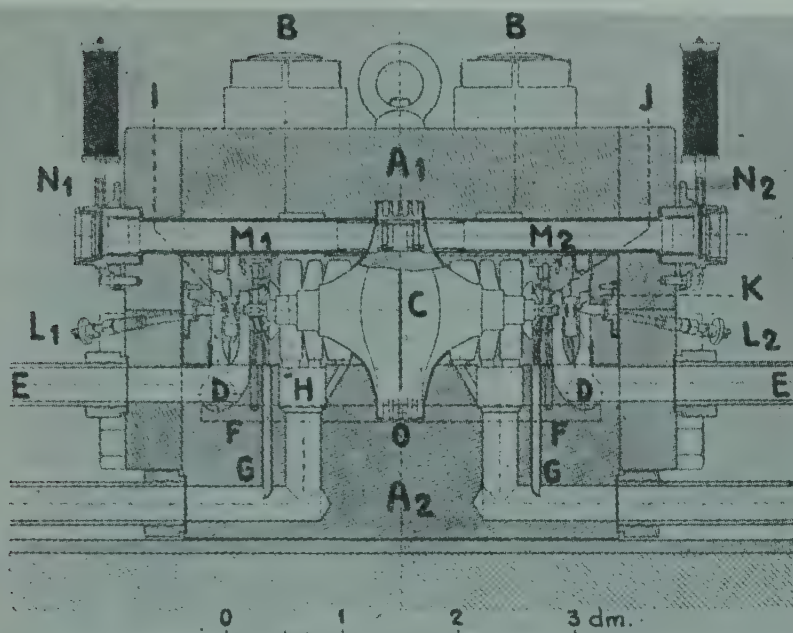
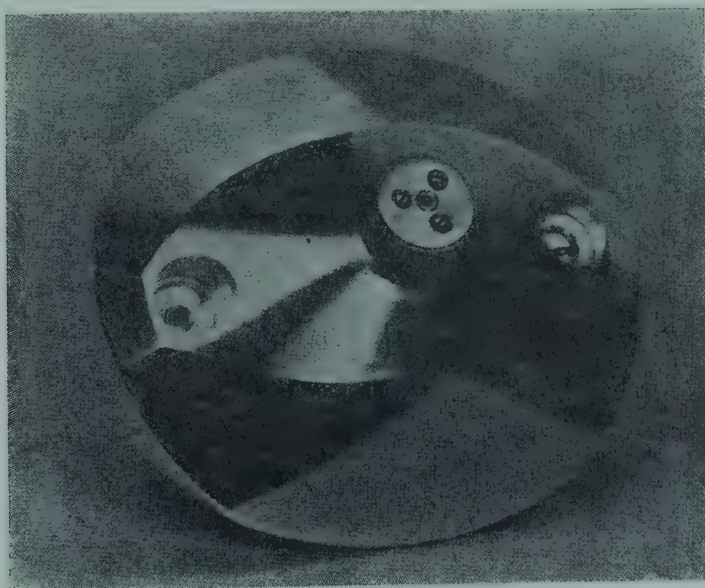


FIG. 52.

A₁, A₂, Centrifuge casing;
 B. Bolts;
 C. Rotor;
 D. Turbine oil inlets;
 E. Turbine oil outlets;
 F. Bearing oil inlets;
 G. Bearing oil outlets;
 H. Drain;
 I. Turbine screws;

J. Main shaft bearings;
 K. Thrust bearings;
 L₁, L₂. Bearing thermocouple;
 L₃. Rotor thermocouple;
 M₁, M₂. Light channels;
 N₁, N₂. Shutters;
 O. Hydrogen inlet;
 P₁, P₂. Coils for speed measurement.
From (1).

FIG. 53. Photograph of rotor. *From (1a).*

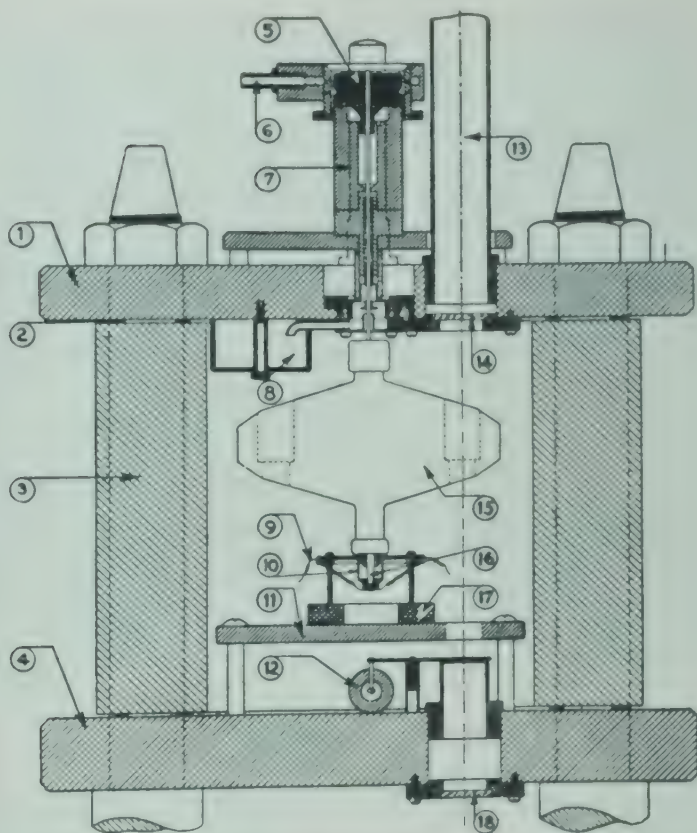


FIG. 54. 1. upper steel end plate; 2. rubber washers for vacuum seals; 3. wall of steel cylinder; 4. lower steel end plate; 5. turbine; 6. air inlet to driving jets; 7. stator; 8. oil container; 9. thermocouple lead; 10. mercury cup; 11. elevated flooring; 12. electromagnetic shutter; 13. metal sleeve connecting with camera bellows; 14. upper quartz window; 15. rotor; 16. tapered steel stem of rotor; 17. circular bakelite base; 18. lower quartz window. *From (1).*

temperature (within 1°C.) over periods as long as 5-6 hours and makes possible measurement near 0°C. Substitution by Beams (6) of an electrical drive for compressed air permits very close control of speed and requires less attention.

For accurate measurements of the sedimentation constant, the temperature of the solution in the cell during centrifugation must be known to within 0.4°C. This is accomplished in the oil-turbine centrifuge by placing a radiation thermocouple very close (0.25mm.) to the surface of the rotor near the opening for the cell. Such a thermocouple has been shown to register the actual temperature of the solution inside the cell (1).

The speed of rotation is usually determined stroboscopically.

Behavior of proteins in the ultracentrifuge: Ultracentrifugal examination of protein mixtures makes possible the resolution of

individual components of different particle size. Failure to find more than a single symmetrical peak by refractive index methods indicates that all of the molecules present are sedimenting at the same rate. This does not necessarily imply that only a single protein is present, since mixtures of different proteins having the same sedimentation constant show only a single component in the ultracentrifuge. Thus fig. 55 shows the ultracentrifugal pattern of a mix-

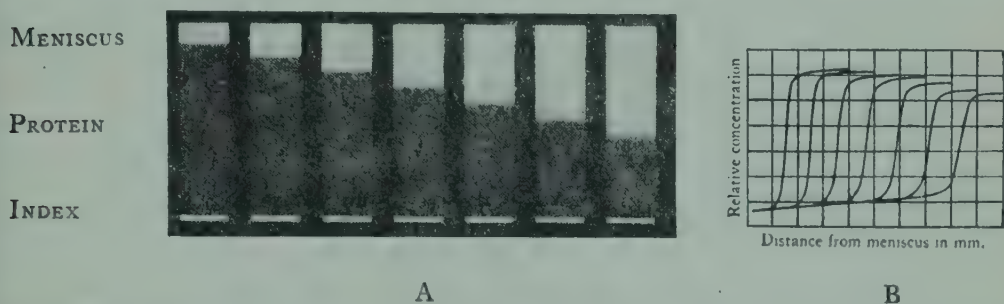


FIG. 55. Sedimentation pictures (A) and photometer curves (B) of a mixture of *Nereis* and *Sabella* blood: centrifugal force 75,000 times gravity: time between exposures 5 minutes. The fact that only one boundary is visible shows that their respiratory proteins, erythrocuorin and chlorocuorin, are identical with regard to sedimentation constant. (Svedberg and Hedenius, 1934) *From (1)*.

ture of *Nereis* and *Sabella* bloods by the light absorption method. Only a single boundary may be seen both from the photographs and the microphotometer tracing, indicating that their respiratory proteins, erythrocuorin and chlorocuorin have the same sedimentation constants (1).

However, Svedberg and Hedenius found that a mixture of *Sepia* and *Octopus* blood shows two boundaries both on the photographs and microphotometer tracings indicating that their respiratory proteins have different sedimentation constants (fig. 56) (1).

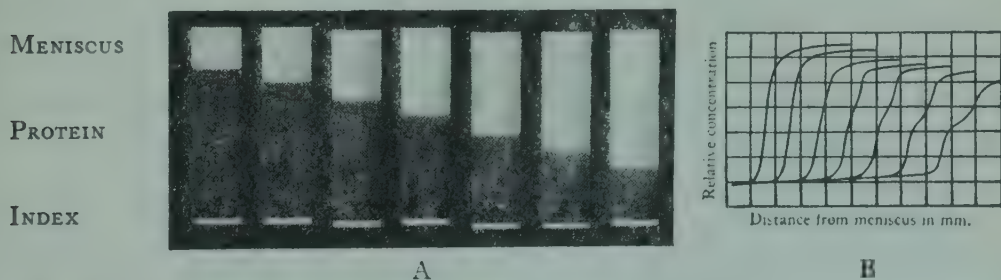


FIG. 56. Sedimentation pictures (A) and photometer curves (B) of a mixture of *Sepia* and *Octopus* blood: centrifugal force 78,000 times gravity: time between exposures 5 minutes. The fact that the boundary is double shows that their respiratory pigments, which are both of the haemocyanin type, have different sedimentation constants, viz. 51.1 and 57.1×10^{-13} . (Svedberg and Hedenius, 1934). *From (1)*.

It must be emphasized that the light absorption method is much less sensitive than refractive index methods for detecting mixtures of proteins in the ultracentrifuge. Thus the lower portion of fig. 57

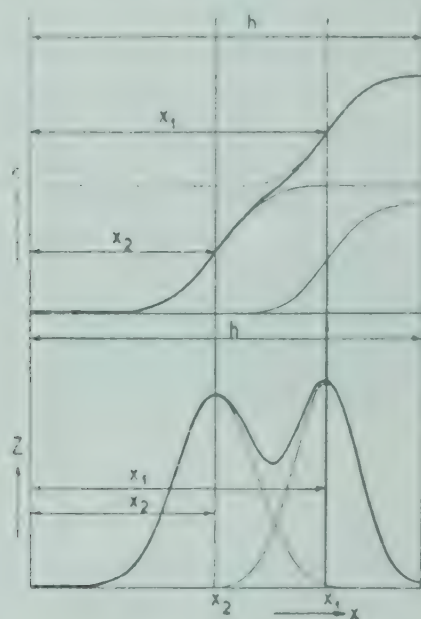


FIG. 57. Schematic drawing showing the sedimentation diagram at the end of a run for a mixture which may just be unequivocally resolved by means of the scale method (lower part of the figure). With the light absorption method (upper part) the diagram barely indicates the presence of two components.

In calculating these diagrams it was assumed that the two components had the same initial concentration, the same specific refractive increment, and same light absorption. The heavy lines represent the curves that would be found in actual experiments. The fine-lined curves show the theoretical curves for the individual components. (h = height of column of solution in the cell, x_1 = distance between meniscus and 50 per cent point for the faster sedimenting component, x_2 = the same for the slower one.) *From (1).*

shows the sedimentation diagram obtained with the scale method at the end of a run for a mixture of proteins. The presence of two components in the diagram is apparent. The upper portion of the figure shows a microphotometer tracing obtained from a photograph of the same solution by the light absorption method; only the barest indication of two components is obtained.

Two series of scale diagrams obtained during the sedimentation of a mixture of serum albumin ($S = 4.5$) and gamma globulin ($S = 7.1$) in varying proportions are shown in fig. 58. The separation of the two components is apparent. From the area under each peak the concentration of each component may be calculated (III, 25). Where complete separation of two peaks is not obtained, the curves

for each component are resolved by geometric construction. These are indicated by the lighter lines in figs. 57 and 58.

Since diffusion takes place while the protein is sedimenting, the boundaries spread as the run progresses. From this spreading the diffusion constant may be calculated. Beckmann and Rosenberg (9) have proposed that agreement between the diffusion constant as measured in the ultracentrifuge and the free diffusion constant be used as a criterion of protein homogeneity (see III, 27).

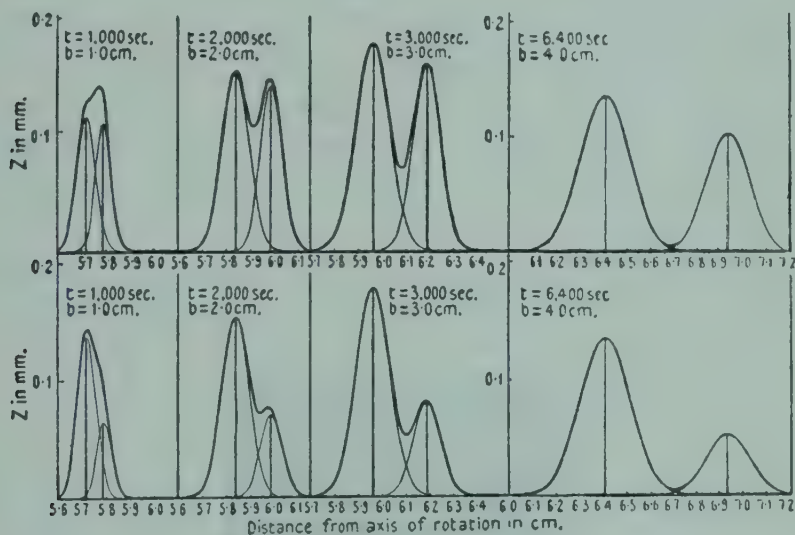


FIG. 58. Illustration of the resolution of the sedimentation curve for a mixture of serum albumen ($S_{20} = 4.5$) and γ -globulin ($S_{20} = 7.1$).

Concentration: upper set of diagrams 0.5 per cent. albumin and 0.4 per cent. globulin, lower set 0.5 per cent. albumin and 0.2 per cent. globulin. Meniscus of solution and bottom of the cell at 5.6 and 7.2 cm. respectively, from the axis of rotation. (In order to save space only the parts including the peaks have been shown in the diagram.) Thickness of the cell:

12 mm. Speed of the centrifuge: 60,000 r.p.m. Temperature: 30° C.

The heavy 'experimental' curves are obtained by addition of the theoretically calculated curves of the albumin and the globulin (the finely traced curves).

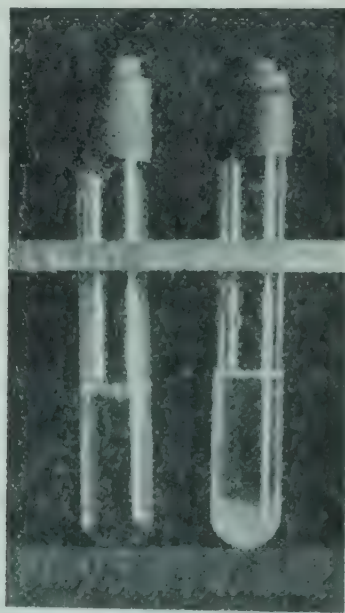
t = time after reaching full speed. b = scale distance. From (1).

Ultracentrifugal analysis has proven of great value as a guide in the purification of proteins in biological fluids such as serum (10), plasma (11), urine (10), milk (12), and other sources of proteins, since it frequently permits identification of any product obtained with a component of the original source material, and may indicate whether any alterations have occurred as a result of the method of purification.

Studies on the effects of pH on the sedimentation constants of

purified proteins have provided information on their stability (I, 13).

By the use of the cell containing the central perforated membrane (fig. 51) it has been possible to determine with which component of a mixture of proteins biologic activity is associated. For example, Type I horse antipneumococcus serum showing an albumin and two globulin peaks was centrifuged until the heavier globulin component was observed to have been sedimented into the lower compartment. When the contents of each compartment were tested with Type I pneumococcal polysaccharide all of the antibody was found in the lower portion (fig. 59) indicating that the antibody was associated with the globulin component of higher molecular weight (14). A detailed account of the ultracentrifugal properties of antibodies is given in (I, 6). Tables summarizing data on the sedimentation constants, molecular weights, and other physico-chemical properties of a large number of proteins may be found in (I, 3, 15, 16).



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FIG. 59. ANALYTICAL DETERMINATION OF SEDIMENTATION

Horse antiserum against pneumococcus Type 1 polysaccharide. Left. Contents of upper cell compartment after addition of polysaccharide. Right. Content of lower cell compartment after addition of polysaccharide. *From (14).*

Limitations of ultracentrifugal analysis: Since refractive index methods are used for measurements of the sedimentation

constant and composition of proteins in the ultracentrifuge, the limitations of these methods, considered in the section on electrophoretic analysis, apply to the ultracentrifugal data as well.

If two proteins in a mixture have the same sedimentation constant, they appear as a single homogeneous component. In complex mixtures of proteins, such as serum, components which comprise but a very small proportion of the total protein are not detectable by optical methods. Thus only two components, albumin and globulin, are generally observed; occasionally, however, a small amount of a heavier globulin component may also be noted. It is futile, however, to attempt to resolve as distinct components substances, such as complement, enzymes, etc., which normally constitute less than 1 per cent of the total protein in serum. Use of the separation cell (8), however, may provide limited information about the sedimentation constants of such components.

Concentrated solutions of mixtures of proteins frequently show an anomalous behavior when examined in the ultracentrifuge, in that the proportion of the slower sedimenting component is increased and that of the more rapidly sedimenting protein is reduced as measured from the sedimentation diagrams. This phenomenon was first observed by MacFarlane (10) who found that when dilute solutions of mixtures of serum albumin ($S = 4.5$) and serum globulin ($S = 7.1$) were examined in the ultracentrifuge, the concentrations of the two components computed from the sedimentation diagram corresponded to their analytical values. However, in more concentrated solutions the relative area of the serum globulin com-

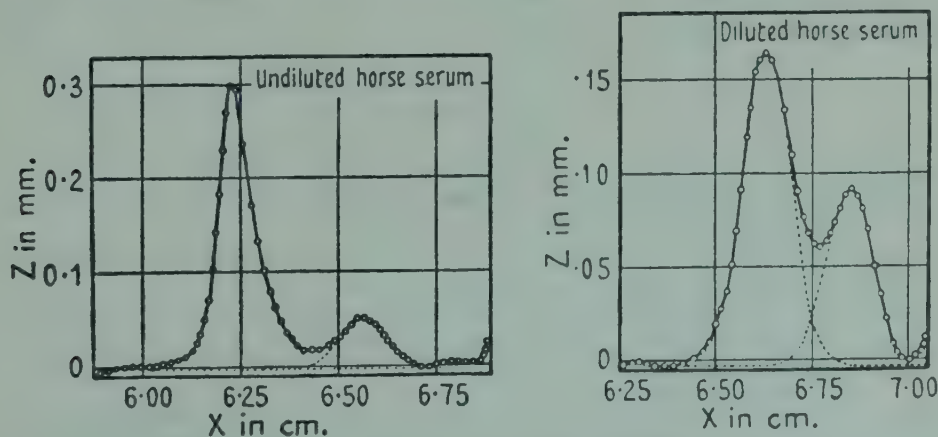


FIG. 60. Sedimentation diagrams by the refraction method showing the effect of dilution on normal horse serum. From (10)

ponent was always low and that of the albumin always high as compared with the analytical values. The ultracentrifugal patterns of undiluted normal horse serum and of the same serum diluted by addition of 3 volumes of 1 per cent NaCl are shown in fig. 60. These observations were extended by Pedersen (17) to other protein mixtures. He obtained similar results and suggested that the lighter molecules caused the heavier ones to dissociate. This phenomenon has been called the MacFarlane-Pedersen effect. It is generally negligible when solutions containing less than 1 per cent protein are studied but constitutes a very large source of error in concentrated solutions. This may also be illustrated by comparison of the sedimentation diagrams for mixtures of 1 per cent serum albumin and 1 per cent lactoglobulin and for 3 per cent serum albumin and 3 per cent lactoglobulin. In the first instance the albumin area measured 55 per cent of the total whereas at the higher concentration it comprised 78 per cent of the total. When a mixture of protamine and serum albumin was studied, a new component of sedimentation constant intermediate between the protamine and albumin was formed which was considered to be an addition compound of dissociated albumin and the protamine. The exact mechanism of this phenomenon is as yet unknown.

Pedersen (18) also emphasizes the importance of salt concentration on the sedimenting boundary. In addition, in human serum at high protein concentration, albumin and globulin and lipids appear to associate reversibly to form a new component (X-protein). Its rate of sedimentation is highly dependent on the density of the solution centrifuged. Pedersen found this component to have a partial specific volume of 0.969.

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CHAPTER 27

DIFFUSION

While it has been recognized for a long time that the specific rate of diffusion of substances in solution is intimately related to the size and shape of their molecules, efficient techniques suitable for the study of the diffusion of proteins and other large molecules were devised only comparatively recently. The development of the analytical ultracentrifuge by Svedberg contributed largely to a revived interest in the diffusion of large molecules since the calculation of molecular weight from the sedimentation constant requires additional information which can be furnished by diffusion data. Thus the diffusion constant, D , appears in the well-known equation [1]:

$$M = \frac{RTs}{D(1 - V\rho)} \quad [1]$$

The techniques of optical analysis used in ultracentrifugation as well as in electrophoresis are also applicable to diffusion and have contributed in large measure to its development.

The purpose of this chapter is to outline the theoretical and experimental principles of diffusion, and to show how diffusion measurements may be applied to the study of proteins. More detailed discussions may be found in reviews by Williams and Cady (2), Kincaid, Eyring and Stearn (3) and Neurath (4).

Diffusion is a manifestation of the thermal energy of molecules, and the speed with which a molecule diffuses under specified conditions of temperature and environment is a function of its size and shape. The specific diffusion rate, or diffusion constant, is defined by Fick's first law which states that the amount of solute, dS , which diffuses in the direction x through a cross sectional area A in time dt is

$$dS = -DA \frac{dc}{dx} dt \quad [2]$$

where dc/dx is the concentration gradient and D the diffusion constant, which is a characteristic property of the solute molecules. The minus sign indicates that diffusion is taking place in the direc-

tion of decreasing concentration. Thus, D measures the amount of solute which would diffuse across unit area in unit time under unit concentration gradient, provided the rate is constant during that interval. The units of D are cm^2 per second. Fick's law is a "dilute solution" law; it is applicable only to solutions which are sufficiently dilute for free diffusion of the solute, that is without interactions of solute molecules. With most proteins this condition is realized below a concentration of about 1 per cent, provided the molecules are not highly asymmetrical.

Molecular motion involves not only translation but also rotation. While important information concerning molecular size and shape can be derived from the study of rotary diffusion (5), the present chapter will be restricted to translational diffusion.

Experimental measurements of the translational diffusion constant are generally obtained by observing the rate at which an in-

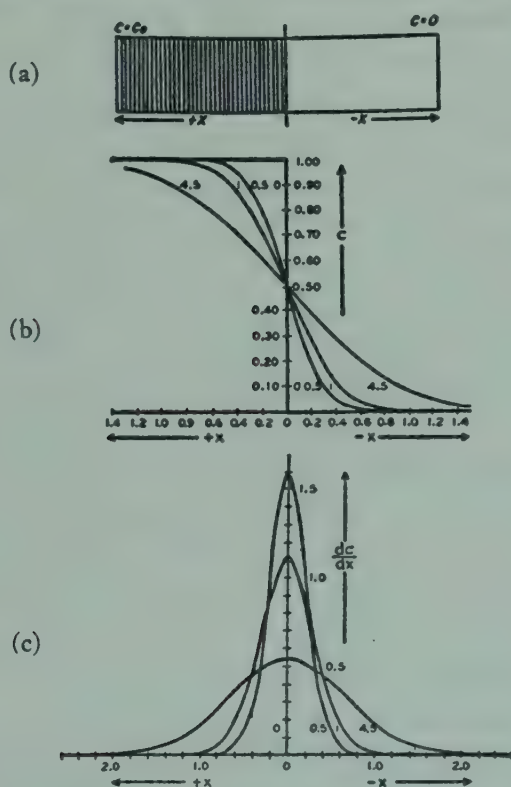


FIG. 61. Relation between concentration, concentration gradient and distance of migration in a diffusion column. (a) Graphical illustration of the diffusion column. (b) Relation between concentration and distance of migration. (c) Relation between concentration gradient and distance of migration. The curves as drawn refer to diffusion times of 0.5, 1, and 4.5 hr. respectively. The whole diagram should be rotated counter-clockwise through an angle of 90° in order to conform with the proper space directions. From (4).

homogeneous system approaches a state of equilibrium. For example, if a column of water is layered over a solution of sucrose in a cylinder, the sugar molecules will diffuse into the water, and provided sufficient time is allowed to elapse, sucrose will be found in equal concentration in all parts of the cylinder. If samples from various levels of the cylinder are analyzed periodically during the diffusion process, the rate at which equilibrium is approached can be estimated. Since withdrawal of samples for analysis changes the composition of the solution and may cause mechanical disturbances, electrical or optical methods of analysis, which do not require removal of samples are preferable. In the study of proteins, a method utilizing absorption of ultraviolet light has been employed by Svedberg and Pedersen (1) and Tiselius and Gross (6), but in recent years it has given way to methods based on refractive index measurements (III, 25) (7), which permit a higher degree of accuracy.

Liquid-liquid boundary method: A solution of the substance in question is placed in a transparent diffusion cell and solvent is carefully layered over it by special techniques and at constant temperature so that a sharp undisturbed liquid-liquid boundary is formed, as illustrated in Fig. 61a. At the start of the experiment (time, $t=0$) the concentration of solute in the lower part of the cell (positive values of x) is c_0 ; in the upper part (negative x) it is zero. The boundary is located at $x = 0$. As diffusion proceeds, the initially sharp boundary becomes diffuse, that is, the rate of change of solute concentration becomes less abrupt as one ascends the diffusion column from positive to negative values of x . It should be noted that concentration changes are confined to the vertical direction since the force of gravity maintains the concentration uniform within any horizontal plane. The concentration at various heights of the diffusion column is measured by optical methods, which permit continuous analysis without disturbing the diffusion process. The ultraviolet light-absorption method used in earlier experiments and discussed in connection with electrophoresis (III, 25) furnished a direct measure of the concentration at any level in the column. With its aid one can obtain concentration as a function of the distance of migration, x . The three curves shown in Fig. 61b represent the relation between concentration and height in the diffusion column at times of 0.5, 1 and 4.5 hours, respectively. It is apparent that all curves intersect at $x = 0$, the position of

the initial boundary, and that at this level the concentration remains constant at $c_0/2$ throughout the entire experiment. Inspection of the curves in Fig. 61b also show a point of inflection at $x = 0$, that is, the rate of change of concentration with respect to x is maximal at this level (cf. slope of curves in fig. 61b). The light-absorption method has, however, been largely abandoned for the refractometric methods (cf. III, 25) which give a direct measure of the concentration gradient or rate of change of concentration, $\frac{dc}{dx}$,

at any level in the column. By plotting $\frac{dc}{dx}$ as a function of x , curves such as those in Fig. 61c are obtained. Diffusion curves are usually presented in this form which is also employed in representing electrophoretic patterns. If diffusion is ideal, i.e., obeys Fick's law, these curves follow the equation

$$\frac{dc}{dx} = \frac{c}{2\sqrt{\pi Dt}} \cdot e^{-\frac{x^2}{4Dt}} \quad [3]$$

in which D denotes the diffusion constant, t the time, and e the base of natural logarithms. This equation can be derived from Fick's first law. Equation [3] is valid only as long as no concentration changes occur at the extreme ends of the cell, and since it is derived from Fick's law, it is, of course, applicable only to dilute solutions where free diffusion takes place. Assuming that the refractive index of the solution is a linear function of the solute concentration, this expression may be written:

$$\frac{dn}{dx} = \frac{n_1 - n_0}{2\sqrt{\pi Dt}} \cdot e^{-\frac{x^2}{4Dt}} \quad [4]$$

where n_1 = refractive index of the solution and n_0 = refractive index of the solvent.

The experimental diffusion curve (Fig. 61c) will agree with the course described by equation [3] or [4], only if the following conditions are met. 1. External forces such as vibration and thermal currents

must be eliminated. 2. To overcome electrical effects due to the charge carried by proteins, measurements are made in the presence of salts, concentrations of about 0.1 ionic strength usually being sufficient to suppress these effects. 3. Dilute solutions should be employed since Fick's law is valid only in dilute solution. Furthermore, equations [3] and [4] hold only for monodisperse systems, that is, solutions containing only a single molecular species.

Calculations: Equation [4], describes a family of curves relating dn/dx and x , with t as a parameter. Three such curves for different values of t are shown in Fig. 61c. The methods of calculating D given below can be used only when the experimental curve obtained at any specified time is an ideal Gaussian distribution or probability type of curve as described by equation [4]. Equation [4] may be simplified as follows:

Replace dn/dx by n' . Substituting specific values x_1 and x_2 for x and corresponding values n_1' and n_2' for n' and dividing n_2' by n_1' , one obtains

$$\frac{n_2'}{n_1'} = \frac{e^{-\frac{x_2^2}{4Dt}}}{e^{-\frac{x_1^2}{4Dt}}} \quad [5]$$

Taking logarithms and solving for D , the following equation is obtained:

$$D = \frac{x_1^2 - x_2^2}{4t \ln \frac{n_2'}{n_1'}} \quad [6]$$

With the aid of this equation the entire course of any experimental curve (fig. 61c) can be utilized for the purpose of calculating D . Any pair of values for x and n' , determined graphically may be used. It is customary to draw several ordinates equally spaced from one another and to calculate a series of values of D from pairs of successive values for x and n' . This type of calculation is known as the method of successive analysis.

A second method, which is less laborious, utilizes the distance u between the two inflection points of the diffusion curve. The or-

dinate of the inflection point is found by dividing the maximum ordinate (at $x = 0$) by \sqrt{e} . The diffusion constant

$$D = \frac{\mu^2}{2t} \quad [7]$$

A third procedure, known as the maximum ordinate-area method, employs the fact that at $x = 0$, the ordinate is maximal and equals

$$n'_{\max} = \frac{n_1 - n_0}{2\sqrt{\pi Dt}} \quad [8]$$

Solving for D and substituting the area A under the curve for $n_1 - n_0$, we obtain

$$D = \frac{A^2}{4\pi t(n'_{\max})^2} \quad [9]$$

The area under the curve is found by graphical integration, with a planimeter.

A statistical method, originated by Pearson (10) and applied to diffusion by Lamm (7) is particularly useful to study ideality of the diffusion process. A detailed description may be found in Lamm's paper (7).

In practice the calculations described above are applied to each of several curves obtained from photographs taken at different times. Since the maximum ordinate (at $x = 0$) is inversely proportional to the square root of t , the progress of the diffusion process with respect to time may be tested by plotting n'_{\max} against $\frac{1}{\sqrt{t}}$. The peaks of all curves should fall on a straight line (4).

Although the inflection point method is simple and least laborious, it suffers from the disadvantage that it affords no test of how closely the experimental curve obeys equation [4]. When the successive analysis method is employed deviations from ideal behavior are readily detected since values of D calculated from different portions of the curve will not agree if diffusion is not ideal.

Calculations also involve certain photographic factors (III, 25). These, and other details, are fully discussed in (7).

Apparatus: The general arrangement for the scale-line displacement method employed by Lamm (7) is shown schematically

in fig. 62. For the "schlieren" scanning or the cylindrical lens methods, different optical systems are needed, but otherwise the arrangements are the same (III, 25).

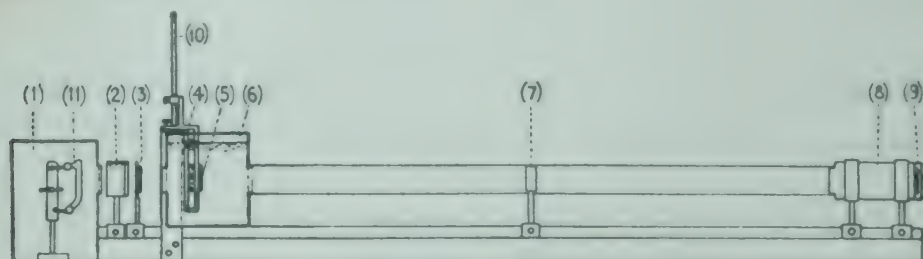


FIG. 62. *Apparatus for diffusion measurements*, consisting of: (1) lamphouse, (11) mercury arc lamp, (2) water filter and light filter, (3) scale with holder, (5) diffusion cell with holder (4) and (10), (6) water thermostat, (7) photographic lens, (8) camera with plate holder (9). *From (7).*

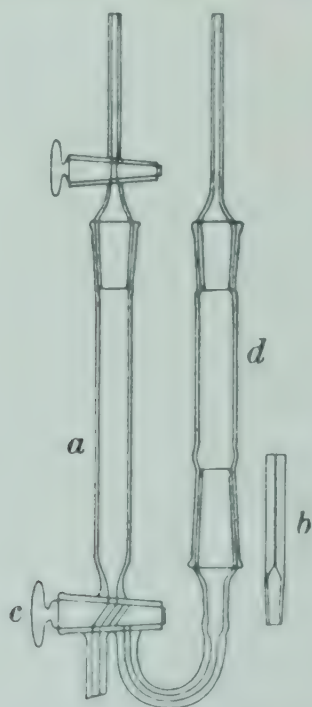


FIG. 63. Cylindrical diffusion cell of Svedberg. The diffusion process takes place in a capillary *b* fits into tube *d*. The solution is introduced by gravity and layered under the buffer. The three way stopcock at *C* is used for levelling. *From (7).*

The circular diffusion cell used in earlier studies by Svedberg (cf. 7) is shown in fig. 63. An improved cell with plane-parallel windows designed by Lamm (7) is shown in fig. 64. It consists of two circular glass discs pressed against the faces of a stainless steel disc with a rectangular vertical slot in the center. A diaphragm of

ebonite, sliding in a horizontal slot, serves to divide the cell into two compartments. A screw arrangement operates the diaphragm from the outside. The cell is filled to a point slightly above the diaphragm with the diaphragm open and allowed to come to equilibrium in the thermostat. The lower compartment is closed off by advancing the

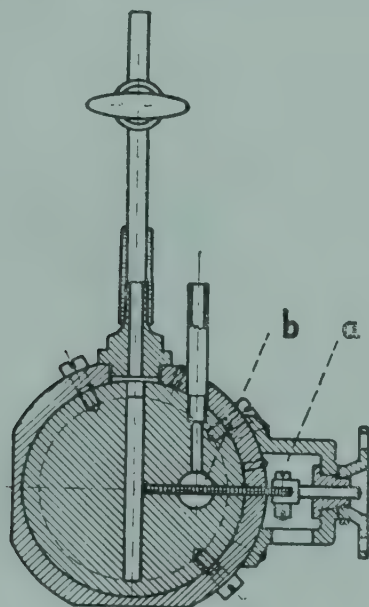
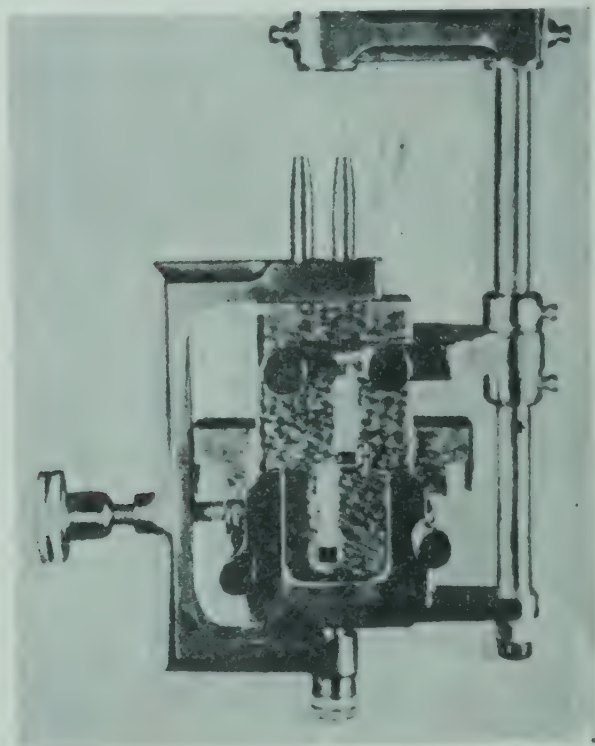


FIG. 64. Metal diffusion cell with slide. Two circular glass plates are screwed against middle pieces of stainless steel, forming a plane parallel cell for the diffusion column. The slide is tightened by wool fat at *a* or by mercury at *b*. From (7).

diaphragm. The upper compartment is rinsed and filled with the buffer against which the solution has been dialyzed and again allowed to come to temperature equilibrium. The diaphragm is then drawn back slowly so that a sharp undisturbed boundary between solution and solvent is formed. Freedom from vibration and thermal equilibrium at each step are, of course essential. A large number of highly satisfactory measurements have been made with this cell in many laboratories.

The Lamm cell suffers from the disadvantage that the column of buffer is displaced vertically several millimeters on withdrawal of the diaphragm. This may be avoided by using an electrophoresis cell (cf. III, 25) but this also is not ideal in that the boundary is formed behind the ground glass plates and has to be moved into view by a compensating mechanism (III, 25). Satisfactory diffusion measurements have, however, been obtained with the Tiselius electrophoresis apparatus (II, 12).

A cell described by Neurath (13) (fig. 65) eliminates the difficulties both of the Lamm cell and the Tiselius electrophoresis apparatus.

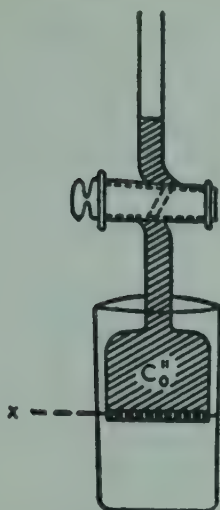


—Courtesy of Williams and Wilkins Company

FIG. 65. Photograph of the Neurath diffusion cell. From (4).

The optical arrangements for recording refractive index gradients in the diffusion column are the same as those described under electrophoresis (III, 25). The Lamm scale-line displacement method (7) is widely employed. The Longsworth "schlieren"-scanning method and the Philpot-Svensson (9) cylindrical lens technique have also been used (III, 25).

Porous disc method: In this diffusion technique, introduced by Northop and Anson (8) and McBain and Liu (14) a sintered glass disc separates the solution from the solvent, as shown in fig. 66. The solution is placed in the cell which is immersed in the solvent, just touching its surface. Diffusion takes place through the pores of the diaphragm. Since the heavier solution is above, gravitational convection currents serve to maintain the concentration uniform within each compartment. The arrangement permits removal of samples without disturbing the diffusion gradient within the mem-



—Courtesy of Williams & Wilkins

FIG. 66. Porous disk diffusion cell of McBain and Liu (14). x denotes the mean position of the boundary, C''_0 the initial solute concentration. From (4).

branes. After varying time intervals, aliquots from each chamber are analyzed and the diffusion constant is calculated from the equation (14a):

$$-\beta D_i t = \ln \frac{c'_f - c''_f}{c'_0 - c''_0} \quad [10]$$

in which β is the cell factor to be determined by calibration with a substance of known diffusion constant, t represents the time, c'_f and c'_0 the final and initial concentrations of the stronger solution, and c''_f and c''_0 the final and initial concentrations of the weaker solution. D_i is the integral diffusion constant representing an average value over a range of varying concentration. If the diffusion constant is independent of concentration, D_i is identical with D , the true differential coefficient. If not, D and D_i are related as follows (15):

$$D = D_i + c \frac{dD_i}{dc} \quad [11]$$

More detailed theoretical discussion may be found in papers by Mehl and Schmidt (15), Anson and Northrop (16) and McBain and Liu (14).

Although this method is simple and rapid, it suffers from the disadvantage that it yields only relative values for the diffusion constant which must be converted into the absolute diffusion constant

by calibration of the cell with a known substance. On the other hand, it permits analysis of small amounts of biologically active substances present in mixtures. The method provided some information about the size of specific polysaccharides (16a).

FACTORS INFLUENCING THE DIFFUSION RATE

Temperature: The variation of the diffusion constant with temperature follows the simple equation:

$$\frac{D_x}{D_y} = \frac{T_x}{T_y} \cdot \frac{\eta_y}{\eta_x} \quad [12]$$

in which D_x and D_y are the diffusion constants at the absolute temperatures T_x and T_y respectively. η_x and η_y are the viscosities of the solvent at T_x and T_y . The factor to convert to D_{H_2O} at 20° from measurements at 0° is 1.9.

Viscosity: Diffusion results are usually corrected to the viscosity of water using the relation

$$D_x = D_y \cdot \frac{\eta_y}{\eta_x} \quad [13]$$

in which η_y is the solvent viscosity and η_x is that of water. It is stated by Neurath (4) that this equation is not strictly applicable above values for 1.1 for $\frac{\eta_y}{\eta_x}$. Neurath found that the diffusion constant of serum albumin in acetate-buffer containing 10 per cent sucrose was $6.43 \times 10^{-7} \text{ cm}^2 \text{ sec.}$ (corrected for the viscosity contribution of sucrose using equation [13]), as compared with a value of 6.99×10^{-7} in the absence of sucrose (17). Measurements on egg albumin by Polson (18) in ammonium sulfate solutions, however, indicated a slight increase in the diffusion constant corrected for viscosity by equation [13]. These discrepancies probably merit further investigation.

The effect of solute concentration: From [2], the constant D should be independent of concentration provided the solution is sufficiently dilute to permit the diffusing molecules to move without mutual interference. In the case of ovalbumin, this condition is realized up to a concentration of 1.4 per cent (18) but on the other hand tobacco mosaic virus shows concentration dependence at

concentrations as low as 0.2 per cent (19). When D is found to vary with concentration, determinations may be made at several different concentrations and results extrapolated to zero concentration, analogous to the procedure followed for sedimentation rates (cf. III, 26; I, 6). This procedure is, of course, valid only if no dissociation occurs in dilute solution.

Polson (18) attempted to relate the concentration dependence of D to the molecular weight. Thus, solutions of large molecules like *Homarus* hemocyanin containing fewer molecules per unit volume show less variation with concentration than do solutions of smaller molecules like lactoglobulin or egg albumin at equal concentrations on a weight basis. While it is obvious from Polson's results that molecular size is important, it would appear that shape plays a role as well. The abnormal behavior of tobacco mosaic virus is probably due to its highly asymmetrical shape. In this connection, Neurath (4) has proposed a "full" viscosity correction which would take into account the viscosity contribution of the protein in a manner similar to that proposed by Lauffer for sedimentation con-

stant measurements (III, 26). Instead of correcting D by $\frac{\eta_{\text{solvent}}}{\eta_{\text{water}}}$

Neurath applies the factor $\frac{\eta_{\text{protein solution}}}{\eta_{\text{water}}}$ but the values thus obtained appear to be too high. On the other hand, when the frictional resistance of the protein molecules is neglected, low values are obtained. An empirical correction equation which appears to be satisfactory is offered by Neurath (4).

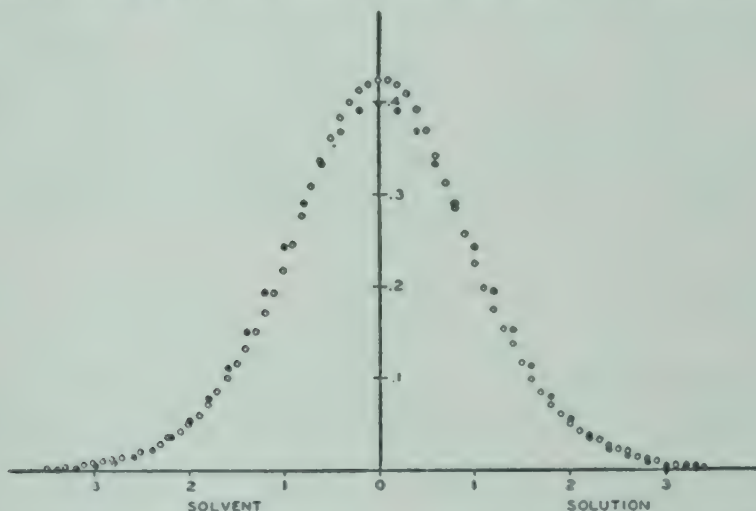
The choice of a suitable concentration thus depends on the nature of the protein. An upper limit of about 1.5 per cent and a lower limit of about 0.2 per cent are imposed by optical considerations. In dealing with a substance which has not been investigated previously, it is advisable to perform experiments at different concentrations.

INTERPRETATION OF RESULTS

It is stated by Neurath (4) that if a solution is monodisperse and diffuses ideally, diffusion constants calculated by equation [6] from any part of the curve, as well as those calculated by equations [7] and [9] should agree to within about 2 to 3 per cent. Furthermore, constants calculated from curves taken at different times should

also agree, provided the time of diffusion is not so long that concentration changes have occurred at the extreme ends of the cell. In addition the same values should be obtained at different concentrations. In case of failure to obtain reasonably concordant results the question arises as to which value is the best. Any attempt to answer this problem is, however, futile, since discrepancies should serve as a warning and attempts should be made to discover the cause of the difficulty. The more common causes for deviation from ideal diffusion are therefore considered.

Non-Ideal, symmetrical curves: If the experimental diffusion curve departs from the course of equation [4], but is symmetrical as illustrated in fig. 67, the reason may be that the material is poly-



—Courtesy of Williams and Wilkins Company

FIG. 67. Comparison of an ideal Gaussian distribution curve with the diffusion curve as obtained from measurements on a 0.8 per cent serum albumin solution at pH 7.6, denatured by heating for 30 min. at 70°C. Polydispersity is most clearly indicated by the difference in maximum ordinates of the ideal curve (full circles) and the experimental curve (open circles). Unpublished experiments by Neurath, Cooper, and Erickson. From (4).

disperse. The use of equation [6] reveals such discrepancies in that identical values of D over all parts of the curve will not be obtained. If equation [7] is employed to calculate a single value of D polydispersity may not be detected. The value of D thus obtained would not represent any one of the components of the mixture but would be a resultant value. Where a material is known to be polydisperse from ultracentrifugal measurements, attempts at purification should precede determinations of the diffusion constant. A method of analysis developed by Neurath (4), however, makes it possible in

certain cases to calculate the diffusion constants of the components of a binary mixture.

In the early days of ultracentrifugation, diffusion constants were calculated from the spreading of the boundary. Although this method has been abandoned in favor of separate diffusion experiments, it should still be valuable when it is desired to determine the diffusion constants of several substances in mixtures of proteins.

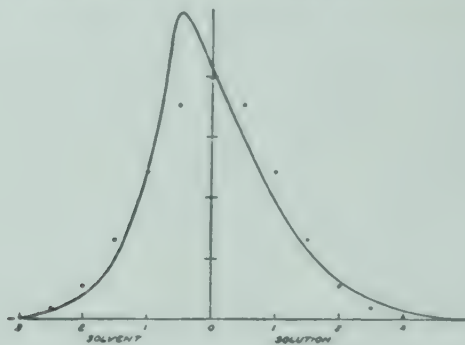
Because of the insensitivity of diffusion curves to polydispersity, an experimental curve which appears to be ideal in all respects cannot be considered good evidence for monodispersity. To be detectable by diffusion measurements, a contaminant must be present in large quantity and its diffusion constant must differ widely from that of the main component. In all respects the ultracentrifuge is the most suitable tool for detecting polydispersities of molecular size. Agreement of the free diffusion constant with the value obtained from boundary spreading in the ultracentrifuge provides a very sensitive test for homogeneity (20).

Drift of D with time: Abnormalities of this sort are apparently widely observed. Many of Polson's (18) and Neurath's (4) determinations on monodisperse proteins show this effect. Bevilacqua and coauthors (21) state that this is often due to the fact that the initial boundary is not infinitely sharp as is required by equation [4]. In that case, the zero time is actually some time before the boundary was formed, and a corresponding correction of t would be necessary. This effect seems to be most serious when compensation has to be used as in the case of the cell of Sevedberg (7) (fig. 63) and the Tiselius electrophoresis cell, and least with a sheared boundary type of cell like that of Neurath (fig. 65).

When diffusion is not free due to the use of too high a concentration, D shows variation with time. A case, studied by Neurath (22) will serve as an illustration. Solutions of protein with rabbit papilloma virus activity exhibit diffusion anomalies in concentrations above 0.2 per cent, as shown in a plot of n'_{\max} against $\frac{1}{\sqrt{t}}$. At 0.3 and 0.5 per cent concentration a straight line is not obtained, indicating a restriction of free diffusion.

For the study of highly asymmetric proteins such as tobacco mosaic virus it would be desirable to perform experiments even below 0.2 per cent concentration but refractive index methods are

not sufficiently sensitive. Bevilacqua (21) has suggested that interferometric methods may overcome this difficulty.



Courtesy of Williams & Wilkins Co.

FIG. 68. Comparison of an ideal Gaussian distribution curve with the diffusion curve as obtained from measurements on a 1 per cent solution of tobacco mosaic virus protein. The dots indicate the position of the ideal curve; the solid line refers to the experimental curve.

From (4).

Asymmetrical deviations: Abnormalities of this type are encountered with extremely elongated proteins, such as tobacco mosaic virus. A diffusion curve of this protein which shows marked skewness is shown in fig. 68. It is clear that in such cases diffusion does not obey Fick's law and it is believed that the extremely elongated molecules become entangled, thus obstructing one another. The rate of diffusion would therefore be greater on the solvent side of the boundary where the molecules diffuse into solvent and as a result the curve will be skewed in that direction.

Diffusion data which show drift with time, asymmetric curves, or other non-ideal behavior may be subject to very large errors. Even when diffusion curves follow equations [3] and [4] results on the same protein studied in various laboratories may vary by as much as 10 per cent.

APPLICATIONS OF DIFFUSION DATA

In dilute solution the diffusion constant is inversely proportional to the molar frictional coefficient, f , that is the force which must act on one mole of the diffusing substance to give it a velocity of 1 cm. per second:

$$D = \frac{RT}{f} \quad [14]$$

where R is the gas content and T the absolute temperature.

For relatively large spherical molecules, moving at constant velocity through a viscous medium, Stokes' law gives the molar frictional coefficient, f_0 :

$$f_0 = 6\pi\eta rN \quad [15]$$

in which η denotes the viscosity of the medium, r represents the radius of the molecule, and N is the Avogadro number. Substituting r in equation [15] by:

$$r = \frac{(3VM)^{1/3}}{4\pi N}$$

in which V is the partial specific volume and M the molecular weight, we obtain:

$$f_0 = 6\pi\eta N \frac{(3MV)^{1/3}}{4\pi N} \quad [16]$$

Rearranging equation [14] into the form:

$$f = \frac{RT}{D} \quad [17]$$

and dividing equation [17] by equation [16], one obtains Svedberg's frictional ratio (f/f_0) or dissymmetry constant f/f_0 :

$$f/f_0 = \frac{RT}{6\pi N \eta D \cdot \frac{(3MV)^{1/3}}{4\pi N}} \quad [18]$$

If the frictional ratio is 1 for any substance, it must consist of compact, spherical and not appreciably hydrated molecules. If f/f_0 is greater than 1, the molecule must be either hydrated or non-spherical, or both. Solving equation [18] for the molecular weight M , one obtains:

$$M = \frac{R^3 T^3}{162 \pi^2 \eta^3 N^2 D^3 V} \cdot (f_0/f)^{1/3} \quad [19]$$

With the aid of equation [19] molecular weights can be determined from diffusion measurements combined with data such as viscosity or dielectric dispersion measurements (5) which yield a value for f/f_0 . Perrin's equation (1) relates f/f_0 to the apparent molecular shape as expressed by the ratio of a major to a minor axis of a prolate or oblate ellipsoid of revolution. As shown in the chapter on

viscosity (III, 28), information on the apparent molecular shape of proteins may be derived from viscosity data. Thus molecular weights can be calculated from diffusion and viscosity determinations on the same substance.

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CHAPTER 28

VISCOSITY

While absolute determinations of the size and shape of large molecules are usually based on ultracentrifugal and diffusion studies it is sometimes possible to derive such information from measurements of viscosity. These are advantageous in that they do not require elaborate apparatus and may be carried out rapidly. Although it is well known that the viscosity of a solution is related to the volume occupied by the solute molecules as well as to their shape, the quantitative theory of viscosity is still relatively undeveloped as compared with ultracentrifugal and diffusion theory. Nevertheless viscosity measurements have proven useful in certain problems, particularly those involving the splitting of large molecules into smaller units, or in the study of reactions accompanied by aggregation. For example, the action of the enzyme hyaluronidase on hyaluronic acid is accompanied by a drop in viscosity and its course may readily be followed by viscosity measurements (1).

Viscosity determinations have served in some instances to control the degree of degradation of biologically active substances. For example, certain specific polysaccharides of the pneumococcus possess a characteristically high viscosity when prepared by mild methods. Degradation resulting from drastic treatment results in a marked decrease of viscosity and in serological activity (cf. IV, 51).

When native proteins are altered by various methods an increase in viscosity is often observed. For example, treatment of egg albumin with urea (2), acid, alkali, or heating (3) produces an increase in viscosity which is believed to be the result of greater molecular asymmetry (4). Certain proteins, like tobacco mosaic virus and myosin which are extremely elongated molecules, behave differently in that treatment with urea results in a decrease of viscosity (4). Such behavior may be explained by dissociation of the extremely long molecules of these proteins into smaller, less asymmetrical units (4).

When large molecules, such as proteins or polysaccharides, are introduced into a solvent it is found that the viscosity of the solution is greater than that of the solvent. The viscosity of the solution,

η , divided by the viscosity of the solvent, η_0 , gives the *relative viscosity* (5), i.e.,

$$\text{relative viscosity} = \eta / \eta_0 \quad [1]$$

Another characteristic, the *specific viscosity* of a solution is defined by the relation:

$$\text{specific viscosity} = \eta / \eta_0 - 1 \quad [2]$$

For spherical solute molecules which are rigid, not solvated, uncharged, and relatively large in respect to those of the solvent, Einstein (6) proposed a simple relation between the specific viscosity and volume concentration, viz.

$$\eta / \eta_0 - 1 = 2.5 \phi \quad [3]$$

in which ϕ is the volume fraction occupied by the solute molecules. From equation [3] it follows that the specific viscosity does not depend on the size of individual spheres but is a function of their aggregate volume.

The viscosity increment v , is defined as follows:

$$v = \frac{1}{\phi} (\eta / \eta_0 - 1) \quad [4]$$

This quantity is a characteristic of the solute, i.e., it represents the viscosity contribution of the solute per unit volume concentration. According to the Einstein equation, v should equal 2.5 for unsolvated spheres. Actually only a few substances have a viscosity increment near 2.5 (7). Most proteins, with egg albumin as a notable exception, exhibit viscosity increments greater than 2.5. This is believed to be due partly to solvation and partly to molecular asymmetry.

With most substances, specific viscosity is a linear function of concentration only in dilute solutions. It is common practice, therefore, to employ the limiting value of v (denoted by $[\eta]$ or $\lim_{\phi \rightarrow 0}$ as ϕ approaches zero. This function is termed the *intrinsic viscosity*. Kraemer (8) uses the symbol $[\eta]$ for the intrinsic viscosity expressed in terms of weight concentration, i.e.

$$[\eta] = \lim_{c \rightarrow 0} \frac{1}{c} (\eta / \eta_0 - 1) \quad [5]$$

The term c denotes the concentration in grams per liter of solu-

tion. Since ϕ equals $\frac{Vc}{1000}$ where V is the partial specific volume, the relation between $[\nu]$ and $[\eta]$ is given by the equation:

$$\frac{[\nu]}{[\eta]} = \frac{1000}{V} \quad [6]$$

The concentration function for proteins is described quite well by the empirical exponential equation of Arrhenius (9):

$$\eta/\eta_0 = K^c \quad [7]$$

in which K is a constant and c represents the concentration.

Treffers (10) has observed that with many proteins the relative fluidity, η_0/η , varies as a linear function of concentration, i.e.,

$$\eta_0/\eta = 1 - Kc \quad [8]$$

Almost all of the proteins for which viscosity data are available obey this relation, some of them up to a concentration of 14 per cent. Several of the cases studied by Treffers are given in fig. 69.

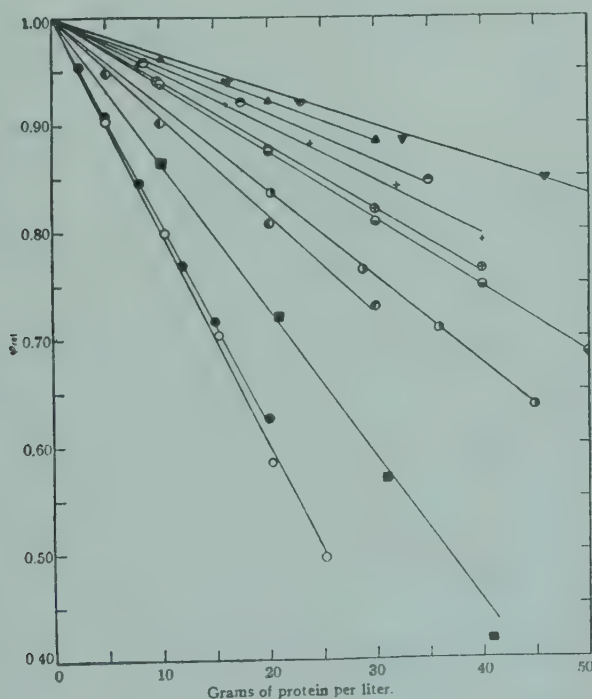


FIG. 69. Fluidities of representative protein solutions. ∇ , oxy-hemoglobin^{6a}; \blacktriangle , CO-hemoglobin⁴; \ominus , homarus hemocyanin⁴; $+$, trypsin^{6b}; \oplus , octopus hemocyanin⁴; \odot , thyroglobulin⁴; \bullet , horse pseudoglobulin, \blacksquare , PII, \circ , PIII (horse globulin fractions)^{2c}; \odot , gliadin⁴; \bullet , beef globulin^{6c}. From (10).

A comparison of the Einstein, Arrhenius, and Treffers equations for viscosity data on egg albumin and horse serum pseudoglobulin (11) is shown graphically in fig. 70. Curves 1 and 2 represent the

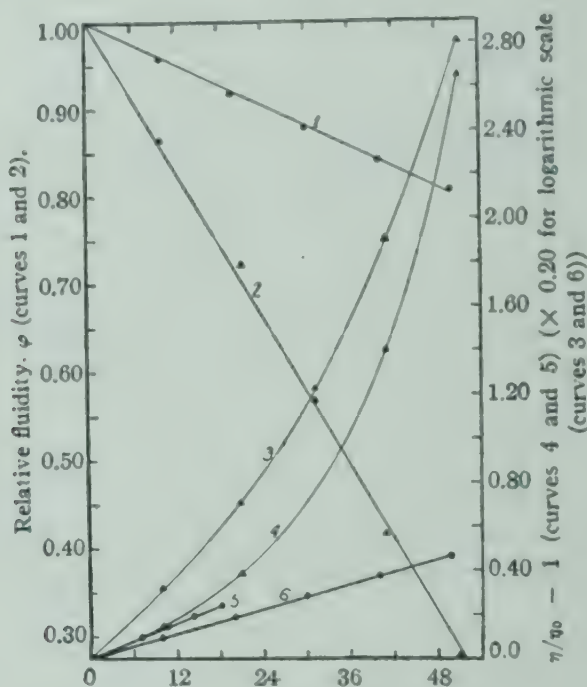


FIG. 70.

fluidities, curves 3 and 6 the logarithms of η/η_0 (linear form of Arrhenius equation), all plotted against protein concentration. Curves 4 and 5 represent the specific viscosity, $\eta/\eta_0 - 1$, plotted against the term KVc^* .

There are several methods of evaluating the intrinsic viscosity $[\eta]$ or $[\eta]$. The most direct way is to plot specific viscosity divided by c as a function of concentration and to determine the intercept of the curve at zero concentration. Alternatively one may employ such empirical relations like that of Arrhenius (9) or Treffers (10) to evaluate the limit (7, 12).

For many substances, including most proteins, the intrinsic viscosity is found to be greater than 2.5. This may be partly due to solvation, because uptake of solvent by the solute increases effective concentration. Part is due to asymmetry, because nonspherical molecules offer more resistance to the flow of liquid, thus giving

*If V is independent of c , the Einstein equation $\eta/\eta_0 - 1 = 2.5 \phi$ can be modified to read: $\eta/\eta_0 - 1 = 2.5 KVc$, where K is a constant.

rise to increased viscosity over that of spherical molecules. The greater the asymmetry of the solute molecules the more pronounced this effect becomes. As a consequence it is possible to obtain information on the shape of the solute molecules from viscosity data.

The orientation of asymmetrical solute molecules also plays a role in determining the magnitude of the viscosity contribution of the solute. If the molecules are not oriented in any particular direction in the flowing solution, the resistance they offer would obviously be greater than if they are aligned in the direction of flow as a result of the stress exerted by the moving solvent. Partial orientation would give rise to an intermediate effect. Whether or not the solute molecules become oriented in the direction of flow depends on the magnitude of the shearing stress of flow tending to orient the molecules relative to the intensity of Brownian motion which tends to maintain a state of random orientation. Intermolecular forces are assumed to be negligible; this is probably true if sufficient salt is present in the solvent to suppress Coulomb forces due to the electrostatic charge of the molecules and when the concentration of protein is not high (13).

The effect of Brownian motion varies with the degree of asymmetry of the molecules. When they are elongated, Brownian motion is less effective in maintaining random orientation than when the molecules are more nearly spherical.

The intensity of shearing stress depends on the velocity gradient of flow maintained in the viscometer. This may be varied in the case of the capillary type viscometer by altering the driving pressure. With the Couette type viscometer (13a) the velocity of rotation may be varied.

With proteins which are not highly asymmetrical, Brownian movement is believed to be sufficiently intense to maintain a state of random orientation during flow under the velocity gradients ordinarily attained. This may be verified by varying the velocity gradient. If the orientation is random, changing the velocity gradient should have no effect on the relative viscosity.

In the case of highly asymmetrical substances like tobacco mosaic virus or myosin it is found that the relative viscosity decreases as the velocity gradient is increased (14). This phenomenon, known as non-Newtonian flow, is believed due to orientation of the solute molecules in the direction of flow. It should be possible to

reach a minimal value of relative viscosity by increasing the velocity gradient until all molecules are aligned in the direction of flow. A study of the relative viscosity of myosin as a function of velocity gradient has been reported by Edsall and Mehl (15).

Recently a relation between the degree of asymmetry and the viscosity increment, ν , has been proposed by Simha (16) for ellipsoidal molecules which are under the influence of sufficiently intense Brownian motion to maintain random orientation. Simha developed two equations, one for rods and another for discs:

$$\text{Rods: } \nu = \frac{J^2}{15(\log 2J - 3/2)} + \frac{J^2}{5(\log 2J - 1/2)} + \frac{14}{15} \quad [9]$$

$$\text{Discs: } \nu = \frac{16J}{15 \tan^{-1} J} \quad [10]$$

The term J denotes the ratio a/b of the axes of an ellipsoid. These relations are the result of a more rigorous treatment than that

TABLE 1
Numerical Values for the Simha Function

J	ν		J	ν	
	rod	disc		rod	disc
1.0	2.50	2.50	20.0	38.6	14.80
1.5	2.63	2.62	25.0	55.2	18.19
2.0	2.91	2.85	30.0	74.5	21.6
3.0	3.68	3.43	40.0	120.8	28.3
4.0	4.66	4.06	50.0	176.5	35.0
5.0	5.81	4.71	60.0	242.0	41.7
6.0	7.10	5.36	80.0	400.0	55.1
8.0	10.10	6.70	100.0	593.0	68.6
10.0	13.63	8.04	150.0	1222.0	102.6
12.0	17.76	9.39	200.0	2051.0	136.2
15.0	24.8	11.42	300.0	4278.0	204.1

Data from (18)

used by earlier investigators (17). For convenience the numerical relations between ν and J are given in table 1.

In addition to the effect of molecular asymmetry, the viscosity increment is also influenced by hydration of the solute molecules. Shape factors are calculated with the aid of equations [9] or [10] on the assumption that the molecules are not hydrated. The same limitation is implicit in shape factors calculated from sedimentation and diffusion constants.

The frictional coefficient, f/f_0 , has been defined in section III, 26 and 27, and its calculation from sedimentation and diffusion constants has been given. Perrin (19) has shown that f/f_0 is related to the ratio of the molecular axes, J , by the equation:

$$\text{For rods: } f/f_0 = \frac{(1 - J^2)^{1/2}}{J^{2/3} \ln \frac{1 + (1 - J^2)^{1/2}}{J}} \quad [11]$$

$$\text{For discs: } f/f_0 = \frac{(J^2 - 1)^{1/2}}{J^{2/3} \tan^{-1} (J^2 - 1)^{1/2}} \quad [12]$$

In table 2 the numerical relations between f/f_0 and J are given for convenience. With the aid of these equations it is possible to obtain f/f_0 from viscosity data. This is done by making measurements of viscosity at several concentrations. The specific viscosity per unit concentration is calculated for each concentration, plotted, and the results are extrapolated to zero concentration to obtain the limiting viscosity increment η . Using Simha's equations J is calculated either for rods or discs and employing the values for the Perrin function given in table 2, f/f_0 is obtained from J . As shown in section III-27 the values of f/f_0 arrived at in this fashion from viscosity data may be combined with the diffusion constant to calculate the molecular weight. A variety of proteins have been studied in this way by Polson (12), Lauffer (7), Neurath and Colloborators (21) and by Mehl *et al* (18). Neurath and Saum (22) have investigated serum albumin in urea solution using viscosity and diffusion. Denatured egg albumin has been studied by MacPherson, Heidelberger, and Moore (3).

Electro-viscous effect: As already mentioned in reference to pneumococcus polysaccharides (IV-51) charged asymmetric molecules exhibit extraordinarily high viscosities in aqueous solutions

TABLE 2

Numerical Values for the Perrin Function

For rods		For discs	
1/J	f/fo	J	f/fo
1.0	1.000	1.0	1.000
1.2	1.003	1.2	1.003
1.4	1.010	1.4	1.010
1.6	1.020	1.6	1.019
1.8	1.031	1.8	1.030
2.0	1.044	2.0	1.042
3.0	1.112	3.0	1.105
4.0	1.182	4.0	1.165
5.0	1.255	5.0	1.224
6.0	1.314	6.0	1.277
7.0	1.375	7.0	1.326
8.0	1.433	8.0	1.374
9.0	1.490	9.0	1.416
10.0	1.543	10.0	1.458
12	1.645	12	1.534
14	1.739	14	1.604
16	1.829	16	1.667
20	1.996	20	1.782
25	2.183	25	1.908
30	2.356	30	2.020
35	2.518	35	2.119
40	2.668	40	2.212
50	2.946	50	2.375
60	3.201	60	2.518
70	3.438	70	2.648
80	3.658	80	2.765
90	3.867	90	2.873
100	4.067	100	2.974

From (20)

containing no salt. This so-called electroviscous effect is due to Coulomb forces exerted by the charged molecules of the solute. It may be suppressed by addition of salt.

Similar effects are also observed in the case of proteins but in most instances a small amount of salt suffices to abolish these electroviscous phenomena (12). It is desirable, however, to verify this by performing measurements at different salt concentrations. In the case of phosphorylated egg and serum albumins it was found that the viscosity varied with pH and in the latter instance it also varied with salt concentration outside the expected range for electroviscous effects (10, 23, 24).

Procedure for measuring viscosity with capillary viscometers (cf 21): The protein solution is dialyzed against salt solution or a buffer of the desired concentration and pH. Before use it

is centrifuged or filtered through sintered glass in order to obtain a clear solution free from sediment, lint or threads, which, if present, might obstruct flow through the capillary.

The viscometer (for details of construction, cf. 21) is suspended vertically in a constant temperature water bath maintained within $\pm 0.05^\circ \text{C}$. An accurately measured volume of protein solution is introduced with a pipette. Care should be exercised to avoid formation of foam, but if any bubbles form, they may be broken by introducing a minute trace of octyl alcohol with the aid of a stirring rod.

After temperature equilibrium is reached the solution is sucked up to a point above the upper mark. It is then allowed to flow and the time elapsed between passage of the meniscus from the upper to the lower mark is measured with a stopwatch. At least four determinations are made, and these should agree within 0.4 second. The time of flow should be such that even this variation should give a precision of less than 1 per cent. The solution is then removed and diluted accurately by addition of solvent. A new series of measurements is then taken. This process may be repeated as many times as desired. Between measurements the viscometer should be cleaned and dried. Finally the viscosity of the solvent is measured.

Calculation: The relative viscosity is obtained using the relation:

$$\eta_{\text{rel.}} = \frac{t_{\text{Solution}}}{t_{\text{Solvent}}} \cdot \frac{d_{\text{Solvent}}}{d_{\text{Solution}}} \quad [13]$$

in which t represents the time of flow and d the density. The *absolute viscosity* in poises is obtained by multiplying the relative viscosity by the absolute viscosity of the solvent.

The calculation of $[\eta]$ may be illustrated with a set of sample data on horse serum albumin:

$$c \quad (\eta/\eta_0 - 1) \quad \frac{1}{c}(\eta/\eta_0 - 1) \quad [\eta] = \lim_{c \rightarrow 0} (\eta/\eta_0 - 1) \quad [\eta] = \frac{[\eta]_{1000}}{V} = \frac{[\eta]_{1000}}{0.75}$$

g per liter	27.0	0.114	0.00423	}	0.00412	5.5
	21.6	0.091	0.00421			
	17.4	0.073	0.00419			
(24)						

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CHAPTER 29

SOLUBILITY

According to the phase rule (1, 2), the solubility of a pure substance in a given solvent under definite conditions of temperature and pressure should be independent of the amount of solid phase present. For example, if increasing amounts of NaCl are added to a fixed amount of water the concentration of the resulting solution increases up to saturation. Addition of more solid to the saturated solution has no effect on the amount of salt in the liquid phase since the excess remains undissolved, constituting a solid phase. Such behavior is generally true only for a pure substance, that is, one which consists of a single chemical individual.

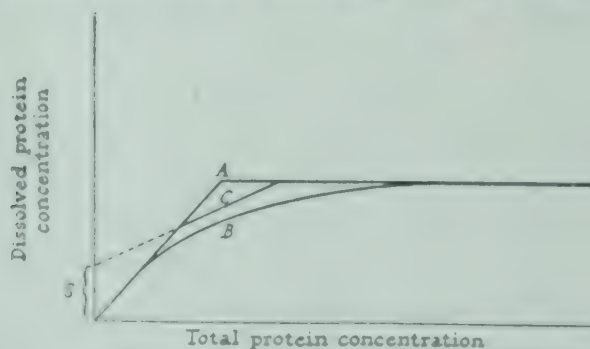
Very few proteins satisfy this criterion of homogeneity. Among those which do not are some which appear homogeneous when examined by ultracentrifugation and by electrophoresis. In most cases the solubility of proteins is found to depend on the amount of solute added, that is, the more solid protein added to the system the greater the amount which dissolves. This has been observed even with crystalline proteins like egg albumin (3) and horse serum albumin (4). Sorensen (4) interpreted this behavior by considering such proteins as systems of reversibly dissociable components i.e., as complexes which break apart or associate as the result of alterations in the composition of the system. Proteins like serum albumin, which deviated a great deal from the behavior demanded by the phase rule were said to possess a large dissociation tendency, while proteins like egg albumin (3) were said to have a small dissociation tendency. Horse hemoglobin (5) was one protein which Sorensen found to exhibit almost constant solubility.

In recent years, however, Northrop and his collaborators (6), working with crystalline enzymes, demonstrated that protein preparations of constant solubility can be obtained by fractionation of impure products. Careful solubility studies have been carried out on chymotrypsinogen (7, 8, 2), trypsin (9) and α , β and γ chymotrypsin (10). Ordinary crystalline pepsin was shown to contain more than one protein (11). One of these was isolated and showed a constant solubility independent of the amount of solid phase present. Other proteins found to be essentially pure from their solubility

behavior include lactoglobulin (12), human hemoglobin (13), horse serum albumin sulfate (14), ribonuclease (15), pituitary luteinizing hormone (16) and lactogenic hormone (17). Kunitz and Northrop (8) have also developed methods of calculation for analyzing the solubility curves of mixtures of proteins.

Since the phase rule applies only to systems which are in equilibrium, it is necessary, in making solubility determinations, to establish that this condition is fulfilled. Measurements should therefore cover a period of time long enough to demonstrate attainment of a steady state, but in addition, it is desirable, whenever possible, to approach the equilibrium state both from the direction of undersaturation and of supersaturation. The study of crystalline serum albumin by Kendall (18) emphasizes the importance of this precaution for it was found in this case that despite the attainment of a steady state the solubility differed depending on the direction from which the state of equilibrium was approached.

Results of solubility measurements are conveniently expressed as a plot of the amount of dissolved protein against the total protein present in the system (cf. 19). In general, three types of curves may be obtained, namely: 1) If the protein is a single chemical individual, the first part of the solubility plot is a line of unit slope which extends to the point where the solution is saturated, that is, the concentration at which a solid phase first appears. Beyond this point the plot becomes horizontal, indicating constant solubility regardless of the amount of solid present in the system (Fig. 71 curve A). At the saturation point, the plot is discontinuous, that is, the transition from the line of unit slope to the horizontal is abrupt.



—Courtesy of Williams and Wilkins Company

FIG. 71. General types of solubility curves. Curve A, results from a single protein component; curve B, results from a solid solution of two or more protein components; curve C, results from a mixture of two protein components. From (19).

2) If several components are present in the protein, and if these form separate solid phases, not a solid solution, the plot will show several sharp breaks, each of which represents a concentration at which a new solid phase appears, indicating saturation with respect to that component. When the plot becomes horizontal the solution is saturated with respect to all components (Fig. 71 curve C). When the constituents of a mixture of proteins form separate solid phases, their separation is accomplished easily. Certain additional information is obtainable from the solubility diagram (assuming ideal solutions). If the line obtained after the first solid appears (line C) is extrapolated, its intersection with the ordinate gives the solubility (S. fig. 71) of the pure component which is appearing as a solid phase. One minus the slope of that line represents the fraction of the pure component in the original suspension (7, 8, 19). 3) If the different components form a solid solution, the solubility curve shows no breaks; there will be a gradual transition between the initial part of unit slope and the final part of zero slope (Fig. 71 curve B). In this case, Raoult's law applies, i.e., the solid phase is richer in the less soluble component than the liquid phase. Purification can therefore be achieved by repeated washing of the solid phase or by repeated crystallization.

The transition region, or regions, require most careful study. After a preliminary series of experiments has furnished the outline of the solubility curve, additional determinations with points closely spaced in the transition region should be made. When the purity of a protein preparation is under scrutiny, the range in which a small amount of solid phase is present deserves most attention, since even mixtures may show constant solubility when the amount of solid phase is large.

While constant solubility, regardless of the quantity of solute, is a good criterion of homogeneity, it has certain limitations. For example a situation may arise in which an impure protein will yield a solid phase or phases which have a composition identical with that of the liquid phase. In such a case, the solubility curve would appear like that of a pure substance. Solubility determinations in several solvents of different pH and ionic strength might eliminate this objection. Such a study has been made by Butler (2) in the case of chymotrypsinogen. In addition, solubility measurements on amor-

phous proteins, while frequently performed, are open to theoretical objections (1,19).

Although constant solubility is possibly the most severe test of protein homogeneity, the best evidence of purity is that which rests on as many tests as possible, including solubility, ultracentrifugation, electrophoresis, serological behavior, amino acid composition (20), etc. (cf. II, 8).

PROCEDURES

Different procedures for solubility determination have been used by various investigators for specific problems.

Solubility of oxyhemoglobins of different species as determined by Landsteiner and Heidelberger (21): Salt-free oxyhemoglobin in the form of a moist crystalline paste was mixed with water at room temperature in a test tube and shaken mechanically for $\frac{3}{4}$ of an hour. The tube was then centrifuged and the supernatant was filtered through a small analytical filter to hold back any crystal fragments. 1.0 ml. portions of the saturated solution were dried to constant weight *in vacuo* at 40-50° C. and the residue weighed. The absence of salt was verified by conductivity measurements.

Differences between the hemoglobins of not too closely related species could be demonstrated by showing that their solubilities were additive, as should be the case for different chemical entities. This was done by addition of oxyhemoglobin crystals of the second species to a saturated solution of oxyhemoglobin of the first species, followed by shaking, centrifugation, filtration and analysis. As a control a second tube containing saturated solution of oxyhemoglobin of the first species was shaken again with additional crystals of the first species. These control tubes usually showed only very small increases of dissolved protein while those to which a second species had been added showed an increase corresponding roughly to the solubility of the second species.

Such was the case, for example, when the solubilities of horse and dog hemoglobin were compared. Comparison of horse and donkey hemoglobin, however, indicated that these species are so closely related chemically that either one can fit into the crystal lattice of the other (isomorphism). While the experiments of Landsteiner and Heidelberger were performed in the region of large

excess of solid phase where solid solutions behave like single substances, a complete solubility curve (figure 71) might have shown that horse and donkey hemoglobin are not identical but formed a solid solution.

Solubility of human serum albumin as studied by Kendall (18): The solubility of crystalline human serum albumin in 2.0 M $(\text{NH}_4)_2\text{SO}_4$ solution at pH 4.9 and 0°C . was determined by approaching the final equilibrium in two ways: first, by allowing the albumin to crystallize from supersaturated solutions and, second, by equilibrating an excess of the crystals with 2.0 M $(\text{NH}_4)_2\text{SO}_4$.

Ten ml. samples of solution containing varying amounts of albumin were mixed with 10 ml. of 4.0 M $(\text{NH}_4)_2\text{SO}_4$ at 15° . The clear solutions were cooled to 0° in an ice bath and seeded with a few crystals. The solutions were stirred for 2 hours and then allowed to stand for 24 hours at 0° . At that time the crystals were centrifuged compactly at 0° and duplicate 1 ml. samples of the clear supernatant solutions were taken for analysis. The crystals were then resuspended in the remaining solution by stirring for 1 hour and kept in an ice bath at 0° . Similar samples for analysis were taken at intervals of several days until constant values were obtained. The pH and the ammonium sulfate concentration were determined at the end of each experiment. Microscopic examination showed that in each case the final precipitate was entirely crystalline and free from amorphous material. The samples for analysis were diluted to 10 ml. with H_2O and heated on a boiling water bath for 10 minutes. The coagulated protein was filtered through a porous porcelain crucible and washed free from $(\text{NH}_4)_2\text{SO}_4$ with hot water. The albumin was then dissolved in 0.1 N NaOH and washed into a Kjeldahl flask. Nitrogen was determined by the micro-Kjeldahl method.

It was found that at least 3 weeks were required to reach constant values. With one exception, the final values were independent of the amount of crystalline protein present.

When crystals were equilibrated with solvent, constant values were reached in about 3 days but the solubility was found to be markedly lower than that noted when the crystals were equilibrated with the supersaturated solution.

It seems evident that since the same solubility was not obtained when saturation was approached from both sides a true equilibrium

was never established between the crystals and the solution, although constant solubility values were obtained. This was attributed by Kendall (18) to the presence of fatty acid in the crystals.

Method of Northrop and Kunitz (8, 10, 19): The technique used by Kunitz (10) in the study of β and γ chymotrypsin may serve for illustration. The protein is recrystallized several times. 10-15 gm. of crystal cake is then suspended in 0.4 saturated ammonium sulfate made up in M/10 acetate buffer of pH 4. The suspension is placed in a 100 ml. Pyrex test tube containing a glass bead, 15 mm. in diameter. The tube is filled completely and closed with a one-hole stopper which is finally plugged with a short glass rod, taking care to exclude air. The tube is agitated slowly on a mechanical rocker so as to keep the bead rolling back and forth for 24 hours. Denaturation due to foaming is avoided by exclusion of air in this manner. The suspension is then filtered and the filtrate is analyzed for enzyme activity and protein concentration. The filtered crystals are then resuspended in fresh 0.4 saturated ammonium sulfate and the procedure repeated several times until the filtrate gives constant values for activity and protein concentration.

After the final washing, the crystals are uniformly suspended in 50 ml. 0.4 saturated ammonium sulfate at pH 4. Increasing amounts of the suspension from 0.1 to 15 ml. are made up to 15 ml. with 0.4 saturated ammonium sulfate and then transferred into 15 ml. test tubes. An 8 mm. Pyrex glass bead is added to each tube. The tubes are closed as above, using one-hole rubber stoppers and short glass rods. After rocking for 24 hours at 10° C., each suspension is filtered through a small Whatman No. 42 filter paper. The clear filtrates as well as the original suspensions are analyzed for enzyme activity and protein concentration. Twenty-four hours is stated to be sufficient to establish equilibrium (10). Results are recorded on a graph plotting protein N per ml. of suspension against protein N per ml. of filtrate.

The use of a preliminary equilibration as described above (8, 10, 19) would result in the removal of more soluble impurities. Therefore the solubility as determined after this equilibration in no sense represents the solubility of the original material. It is preferable, as noted by Herriott (19), to dialyze the material against the solution to be used to attain salt equilibrium.

The review article by Herriott (19) contains a full discussion of

the usefulness of solubility measurements in the purification of proteins.

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CHAPTER 30

ULTRAVIOLET ABSORPTION SPECTRA

Examination of products obtained in the course of chemical fractionations for their specific absorption of ultraviolet light may frequently provide important information as to their composition or constitution. In some instances, absorption in the ultraviolet may be useful for quantitative estimation of various substances.

Proteins show characteristic absorption at 2700 to 2900 Å with a maximum at about 2800 Å (1-3). This absorption has been attributed chiefly to the content of aromatic amino acids especially tyrosine and tryptophane (2), each of which shows three bands in the vicinity of 2700 to 2900 Å (table 1) (4-8).

TABLE 1

Table of Wavelengths of Narrow Bands in Proteins and Aromatic Amino Acids

Serum albumin.....				2533	2583	2613	2645		2688	2733	2788	2847	2900
Egg albumin.....				2532	2587		2650		2680	2742	2799	2855	2923
Thyroglobulin.....				2534	2581	2614	2645		2682	2743	2796	2841	2909
Euglobulin.....			2487	2529		2616	2640		2680	2749	2795	2849	2915
Pseudoglobulin.....					2587		2649		2691	2747	2794	2849	2916
Pneumococcus antibody..					2591		2649		2685	2768		2850	2911
Gelatin.....				2529	2584		2644		2679	2745		2839	
Insulin.....				2530	2586		2645		2683	2766		2839	2898
Tyrosin.....									2672	2747		2816	
Tryptophane.....									2694		2794		2888
Phenylalanine.....	2366	2418	2466	2517	2574	2606	2635	2671	2714				

From (8)

By the use of a continuous spectrum such as is obtained with the hydrogen discharge tube, a number of sharp but weak bands may also be observed in the region from 2300 to 2700 Å. This fine structure is apparently due to phenylalanine (5-8). No aliphatic amino acids have been found to exhibit any specific absorption bands from 2300 to 3000 Å. Histidine and cystine, however, have been found to show some generalized or non-specific absorption in this region (8, 9, 10).

Only the broad absorption band at 2800 Å (fig. 72) is satisfactory for quantitative estimation of protein concentration. Use was made of this specific ultraviolet absorption in following the movement of boundaries in the ultracentrifuge and in electrophoresis (III, 25, 26).

With increasing pH, the absorption band of tyrosine shifts toward longer wave lengths and its intensity is increased (11). This has been attributed by Stenstrom and Reinhard (11) to dissociation of the tyrosine hydroxyl group and Crammer and Neuberger (12) have used the absorption curves at varying pH to calculate the hydroxyl dissociation constant of tyrosine.

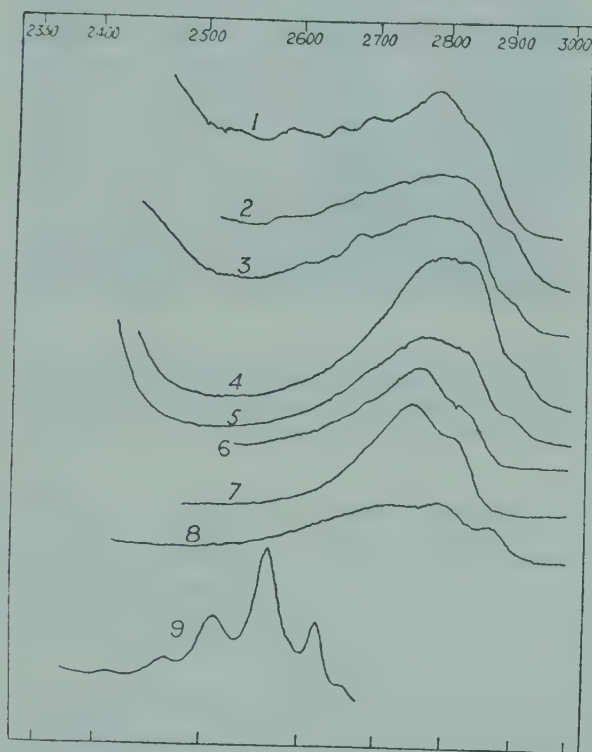


FIG. 72. Microphotometric curves of ultraviolet absorption of proteins and aromatic amino acids. (1) Crystalline horse serum albumin. (2) Hog thyroglobulin. (3) Horse anti-pneumococcal antibody concentrate (Felton solution). (6) Crystalline insulin (pH 4). (7) Tyrosine. (8) Tryptophane. (9) Phenylalanine. *From (8).*

Figure 72 shows a series of absorption curves made as microphotometer density tracings of a number of proteins and of tyrosine, tryptophane and phenylalanine and table I gives the positions of the various bands reported for a number of proteins and amino acids (8). A photograph showing the fine structure of the absorption bands of phenylalanine is given in figure 73 (13). Holiday (14) has employed comparison of extinction measurements at 2800 Å and 3050 Å with known mixtures of tyrosine and tryptophane for estimating the tyrosine and tryptophane contents of unknown solutions and of proteins at constant pH.

Nucleic acids show very strong absorption in the ultraviolet due to the purine and pyrimidine portions of the molecule. The absorption maximum occurs at 2600 Å. The intensity of absorption of nucleic acids is very much greater than that of the proteins, as has been pointed out by Caspersson (15), and the detection of even

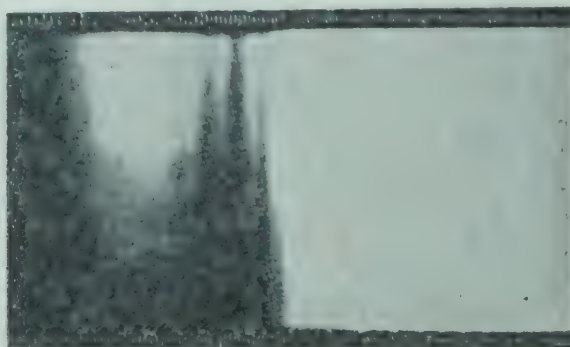


FIG. 73. Spectrogram of a 0.0061 M solution of d-l-phenylalanine showing bands at 235.8, 241, 246, 251.3 256.5, 260.5, 263.2, 266.5. The photograph was obtained by moving the photographic plate past the spectral image at a logarithmically increasing rate and shows the relative intensity of each band. *From (13).*

small amounts of nucleic acid in proteins is readily accomplished spectrographically. The relative absorption intensity of 0.5 per cent sodium desoxyribonucleate as compared with 0.5 per cent serum albumin, histone sulfate and with 5 per cent protamine sulfate is illustrated in figure 74 (15).

Principles of ultraviolet spectrophotometry: If a solution of a protein contained in a tube with quartz end-plates is placed in the path of a beam of ultraviolet light, the intensity of the light after passing through the solution (I) will depend upon the intensity of the light after passing through an equivalent column of solvent (I_0), the thickness of the column of solution (l) and the protein concentration in mg./ml. (c) as given in the following formula (Beer-Lambert Law):

$$\log_{10} \frac{I_0}{I} = kcl \quad [1]$$

where k is a constant called the specific extinction. The value of $\log_{10} (I_0/I)$ is also known as the density (d) or the extinction (E). The constant (k) is dependent upon the characteristics of the material and wave length of light used. When the molecular weight of the substance is known, the molecular extinction (g) may be cal-

culated by multiplying k by the molecular weight. Substances whose molecular weights are unknown may be compared by determining the value of E for a 1 per cent solution and with a column of solution 1 cm. in thickness.

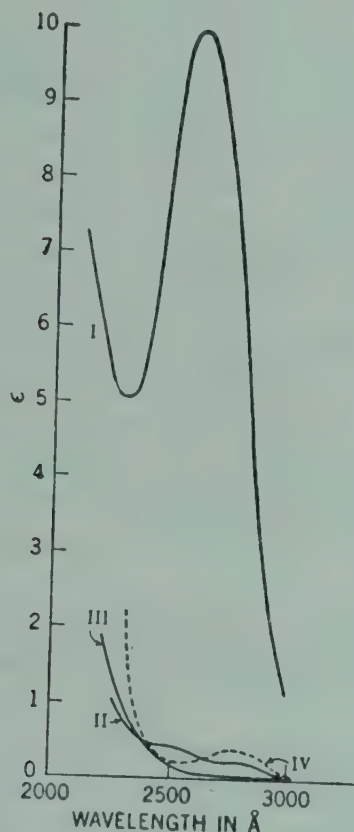


FIG. 74. Absorption curves of I. 0.5% Sodium thymonucleinate; II. 0.5% Histone sulfate; III. 5% Protamine sulfate; IV. 0.5% Serum Albumin (dotted). From (15).

Apparatus: Several ultraviolet spectographs and spectrophotometers are available commercially. Descriptions of many of these and of other equipment may be found in (16). Two of the most convenient types are the Hilger "Spekker" spectrophotometer and the Beckmann photoelectric spectrophotometer.

In the "Spekker" spectrophotometer, light from the source is split into two beams by two quartz rhombs. One of these beams (upper) passes through the cell containing the absorbing solution, while the lower beam passes through a similar cell containing the solvent. The two beams are then directed into the slit of a spectrograph and photographed simultaneously as a pair of adjacent spectra. The instrument also contains a wave length scale which is photo-

graphed at the top and bottom of each plate. The amount of light after passage through the solution is compared with the intensity of light after passage through the solvent. The latter may be varied by a diaphragm which is controlled by a rotating drum calibrated to read the density, $\log (I_0/I)$, directly.

In making measurements with the "Spekker" instrument, a series of 16 to 24 pairs of spectra are photographed on a single plate, each corresponding to a different setting of the drum ($\log I_0/I$). After developing the plate the position of the points where the solution and solvent spectra are of equal intensity on each pair of spectra is determined. These "match points" may be indicated by spots on the plate and serve to delineate the position of the absorption band. The extinction, $\log (I_0/I)$, of each of these points of equal intensity from the drum settings is then plotted against the wave length of the match points to give the absorption curve.

If the lower line of a pair of spectra is dark all the way across the plate for the lowest density, the solution is too dilute. If it is clear for a good portion of the range at the highest density, the solution is too concentrated.

The hydrogen discharge tube furnishes a very advantageous light source since it provides a spectrum which is nearly continuous from 2000 to 3500 Å.

The Beckmann spectrophotometer is a photoelectric instrument with a range of from 2,000 Å in the ultraviolet to 10,000 Å in the infrared. It is very readily used for the quantitative estimation of substances which absorb in the ultraviolet. A calibration curve of the extinction of known amounts of material at a wave length in the absorbing region is prepared, the extinction of the unknown solutions measured, and their concentrations computed from the curve, as is done with the ordinary photoelectric colorimeter. With protein solutions care should be taken to make all measurements at the same pH (11,12).

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CHAPTER 31

TITRATION OF PROTEINS WITH ACID AND ALKALI

According to the concepts formulated by Bronsted, an acid is any substance which can give up a proton, for example, CH_3COOH or the NH_4^+ ion; a base is any substance capable of combining with a proton, for example, hydroxyl ion, the CH_3COO^- ion, or ammonia (NH_3). When an acid like acetic acid loses a proton, it becomes a base, namely CH_3COO^- , or when the base NH_3 takes on a proton, it becomes an acid, that is NH_4^+ . Pairs like CH_3COOH and CH_3COO^- are spoken of as conjugate acids and bases.

The pK of a conjugate acid-base pair is the pH at which the concentrations of the acidic and basic forms are equal. For example, the pK of acetic acid is about 4.7. Therefore, the concentration of unionized CH_3COOH equals that of CH_3COO^- at pH 4.7. Below pH 4.7 the concentration of CH_3COOH is greater than that of CH_3COO^- while above pH 4.7, the opposite is the case. With ammonia (pK 8) the situation is quite similar. At pH 8, the concentration of NH_3 equals that of NH_4^+ . Below pH 8 the acidic form, NH_4^+ , predominates, while above pH 8, the basic form, NH_3 , is present in larger amount (cf. appendix).

Since the amino acids of which proteins are composed are bound in peptide linkage, most of their α -carboxyl and amino groups do not function as acids or bases between pH 1 to 13, the range over which titration of proteins is generally studied; nor do the amide groups of glutamine and asparagine bind hydrogen ion within these limits. The bulk of the acid or base binding capacity of proteins is contributed by those side chain carboxyl groups of glutamic and aspartic acid which are not in amide linkage, by the phenolic group of tyrosine, the sulfhydryl group of cysteine, the imidazolium group of histidine, the ammonium group of lysine and the guanidinium group of arginine. The few α -carboxyl and α -ammonium groups which are usually free at the end of a peptide chain may also bind acid and base.

Following Bronsted (1), these different radicals which are capable of binding and dissociating hydrogen ion may be considered as acid and conjugate basic groups. Each pair may be characterized by its acidity constant, expressed as pK (Cf. table 1.). While the pK

values of most of these groups, when combined in peptides of known structure, have been determined quite precisely, their acidity may possibly be altered by adjoining groups in the protein.

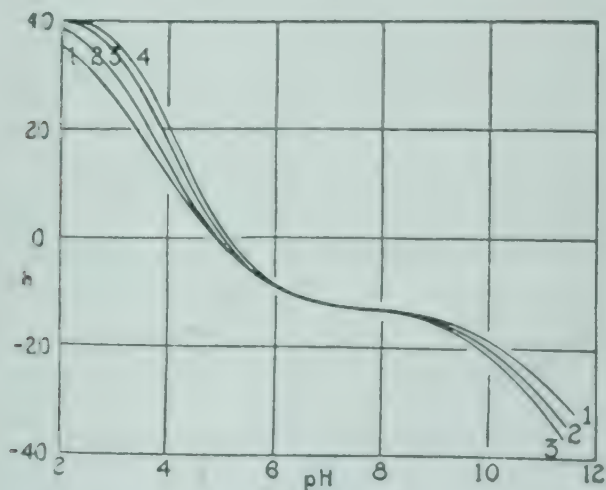
A titration curve is a convenient graphical way of describing the reaction of a protein with acids and bases. The coordinates of the graph are pH and a quantity called h , which is a measure of the hydrogen ions bound or dissociated by the protein. Values of h may be given as equivalents of H^+ per gm of protein or per mol when the molecular weight is known. In a continuous titration, increasing amounts of acid or base are added to a solution of the protein, and the pH is measured after each addition. Alternatively, a protein may be titrated discontinuously by preparing a series of reaction mixtures of a fixed quantity of protein and variable amounts of acid or base. The pH of each mixture is determined. The amount of acid or base bound is calculated by taking the difference between the total amount of acid or base added, and the quantity of free acid or base at that pH. The free hydrogen or hydroxyl ion may be obtained by measuring the amount of acid or base required to bring a protein-free solution of similar electrolyte content to the same pH. The acid or base bound by the protein is then plotted as a function of pH. In strongly acid or alkaline solution the free hydrogen or hydroxyl ion concentration becomes quite large; in these regions the amount of bound acid or alkali therefore represents a small difference between two rather large values, and therefore cannot be estimated accurately. This is one of the factors which limits the accuracy of the titration curve outside the range from pH 2 to 12. Since the maximum acid or base binding capacity of many proteins is not reached within these limits, a convenient point of origin for the scale of h is the isoionic point (pI_i) which may or may not be identical with the isoelectric point pI_e , as determined by electrophoretic measurements. pI_e is defined as the pH at which the net charge of the protein is zero, that is, the number of positive charges equal the number of negative charges. At the pI_e a protein does not migrate in an electric field. pI_i , on the other hand, is defined as the pH at which the number of hydrogen ions dissociated from the acidic groups equals that combined with the basic groups. If the protein combines with no ions other than hydrogen ion, pI_i is identical with pI_e .

In the case of proteins like certain globulins which may be pre-

pared by precipitation from practically salt-free solutions, the pH of the redissolved precipitate corresponds closely to pI_i , provided the protein concentration is relatively high and the salt concentration very low, and the pH values are not outside the limits 4.5 and 9.5 (4).

When proteins are prepared by salting out, ions other than H^+ or OH^- may be combined with the protein and it may be necessary to resort to indirect means for determining pI_i such as electrodialysis or dialysis against dilute ammonia, followed by analytical determination of the latter. By addition of an amount of acid exactly equivalent to the ammonia, the protein is brought to a pH value which is taken as pI_i (5).

The titration curves of most proteins appear quite similar; they differ mainly in slope at different pH values (fig. 75). Generally two regions of strong buffering action may be seen; one lies between pH 2 and 5, while the other extends beyond pH 8. The range between pH 5 and 8 is generally steep, indicating that buffering capacity is relatively low. Since the pH regions within which different groups dissociate merge gradually and overlap in some cases, it is not a simple matter to resolve a titration curve into its constituent parts due to the different protein dissociating groups.



Courtesy N. Y. Academy of Sciences

FIG. 75. Dissociation curves in varying $[KCl]$.

Curve 1 - 0.033 M KCl. Curve 3 - 0.667 M KCl.

Curve 2 - 0.133 M KCl. Curve 4 - 2.38 M KCl.

from (6)

In strongly acid solution a protein carries its maximum positive charge, that of the cationic groups (ammonium, guanidinium,

imidazolium). The anionic groups (carboxyl, sulfhydryl, phenolic) are uncharged in strongly acid media. Addition of alkali first produces dissociation of protons from the carboxyl groups, resulting in a decrease of net charge but an increase of total charge. The buffering capacity of the protein between pH 2 and 5 is mainly due to carboxyl groups. Further addition of alkali probably affects the imidazolium groups next since these have pK values ranging from 5.6 to 7.0 (table 1). Beyond pH 7 several ammonium groups begin to lose protons, but the pK of the ϵ -amino groups of lysine, which constitute the bulk of the ammonium groups is between 9.4 and 10.6. Phenolic and sulfhydryl groups lose their protons in the vicinity of pH 9 to 11, while the guanidinium groups are strongly basic and are not completely dissociated even at pH 13. As mentioned above, this is outside the limits within which accurate titrations can be performed. Therefore it is generally not possible to reach maximum base binding capacity. On the acid side a maximum is often attained near pH 1.5 or 2.

Titration of proteins in the presence of formaldehyde (1-8 per cent) yields curves which indicate increased buffering between pH 6 and 8, and which show a well defined break near pH 8.5. The effect of formaldehyde appears as a lowering of the pK range of ammonium groups from about 9 to 11 to approximately 6 to 8. At pH 8.5 these groups would therefore be in the alkaline form so that a break appears in the curve at this pH. Accordingly the number of free amino groups may be determined by titration to pH 8.5 in the presence of formaldehyde.

Some stoichiometric deductions derived from titration curves may be illustrated from the study of egg albumin by Cannan, Kibrick and Palmer (6). The total acid binding capacity of egg albumin is approached near pH 2, where the value of h equals 41 equivalents per 45000 grams of protein. This quantity represents the total cationic groups (imidazolium, ammonium, guanidinium) per mole since in strongly acid solution these are the only charged groups and their number equals the net charge (h). On the alkaline side no maximum value of h is reached at pH 12, the practical limit of accurate titration. This is in accord with the pK range of guanidinium groups (11.6 to 12.6) which indicates that some guanidinium groups are still charged at pH 12 to 13. At pH 8.5 the difference in h between titrations performed in the presence and

absence of formaldehyde equals 22. This value is believed to be a measure of the amino groups in the protein which react with formaldehyde (6).

The contribution of imidazolium groups may be estimated after Wyman (7) from the characteristic heats of ionization. This leads to a value of 5 such groups for egg albumin.

The number of guanidinium groups may be calculated by subtraction of the 22 ammonium and the 5 imidazolium groups from the total of 41 cationic groups; leaving 14 guanidinium groups.

Since titrations in the presence of formaldehyde yield a maximum base binding value of h equals 37 near pH 8.5, the net charge at this pH is 37, which corresponds to the difference between the negatively charged carboxyl groups and the positively charged guanidinium groups. Since the former are completely ionized at pH 8.5, and since the latter do not begin to lose protons until at least pH 11 is reached, the value 37 should be a quantitative measure of carboxyl minus guanidinium, i.e. number of carboxyl groups minus 37 equals 14. Therefore, total carboxyl should equal 51.

Agreement of these values with those derived from amino acid analysis for egg albumin is quite good. Lysine accounts for only 15 amino groups. Perhaps the remaining 7 amino groups represent terminal α -amino groups. Amino group estimations with the nitrous acid reaction (cf. III-14) agree fairly well with those derived from titration curves. Free carboxyl groups plus phosphoric acid groups total 47, only 4 short of 51. Perhaps this small difference is due to losses in the analytical isolation of dicarboxylic amino acids from protein hydrolyzates. (4).

Sources of error and limitations: 1) Measurements of free hydrogen and hydroxyl ion concentrations at different pH values for the purpose of subtracting these quantities from the total acid or base added, involve the assumption that the protein ions do not affect the activity coefficient of hydrogen ion and that they exert no influence on the liquid junction potential. Errors from these sources are likely to be minimized if titrations are performed in solutions containing a neutral, uni-univalent electrolyte, like KCl, rather than in a system containing only protein and acid or base.

2) The solvent for the protein should contain no substances which combine with or dissociate hydrogen ion within the pH range of the titration curve. This may be accomplished by dialysis

of the protein solution against a solution of KCl followed by removal of CO_2 in a desiccator over KOH solution of the same normality as that of the KCl solution. During the titration CO_2 must be kept out.

3) If irreversible reactions between the protein and acid or alkali occur they may in some cases lead to changes in the number of acid or base binding groups. Reversibility of the reaction may be checked by back titrating. Irreversible reactions may also be manifested by drift of pH with time. Absence of drift does not, however, necessarily indicate reversibility since some irreversible reactions may be quite rapid. When difficulties of this sort are encountered, it would appear preferable to employ the discontinuous titration technique.

Salt effects: The form of the titration curve of any given protein depends somewhat on the ionic strength and the nature of the salt in the solvent. Qualitatively the effect of increasing salt concentration is to render more alkaline those solutions which are on the acid side of pI , while those alkaline to pI become more acid. These phenomena in the case of egg albumin have been studied by Soerensen, Linderstrom-Lang and Lund (5), and Cannan, Kibrick and Palmer (6). The latter authors also investigated the behavior of β -lactoglobulin (8) in this respect. An electrostatic theory has been advanced by Linderstrom-Lang (9) and by Cannan (6). In the case of wool protein, which exhibits similar effects, Steinhardt (10) explained the data on the hypothesis that anions as well as hydrogen ions are bound by the protein in the acid range of the titration curve.

Besides egg albumin, another crystalline protein which has received careful study is β -lactoglobulin. As in the case of egg albumin, good agreement has been obtained between stoichiometric analysis of titration curves and direct amino acid analysis for the dicarboxylic acids, and for histidine and arginine. The value obtained for lysine did not account for total titratable ammonium groups, possibly because of the presence of terminal α -amino groups.

In the study of protein derivatives such as phosphorylated egg albumin or serum albumin (cf. IV-46) the study of titration curves should yield information on the mode of attachment of the substituent groups. For example, if they are bound to lysine there would be a decrease of titratable ammonium groups.

PROCEDURE

Dialyze about 6 ml. of a 1 per cent protein solution against several changes of 0.15 *M* KCl solution in the cold. Transfer the solution to a beaker and keep in a desiccator over 0.15 *M* KOH for one day. After removal of CO₂ in this manner the protein solution should not be exposed unnecessarily to the atmosphere.

The titration system of MacInnes (13) as employed by Grabar and Treffers (11) consists of two glass electrodes and a vacuum tube potentiometer. The electrodes are of the condenser type into which the solution is drawn by suction. One of these is immersed directly in the protein solution, while the other is filled with a phosphate buffer of about pH 6. The end of the latter electrode is drawn out to a very fine tip, which is immersed in the protein solution. In this manner, the latter electrode is kept at a constant pH while the former records the changes occurring in the protein solution. The potential difference (e.m.f.) between the two electrodes is measured at each step of the titration. The system does not include a calomel electrode except that the initial and final pH of the protein solution before and after addition of acid or alkali is measured with a glass electrode-calomel cell as usual. During the titration proper, the difference of e.m.f. between the two glass electrodes is measured after each addition of acid or alkali.

The titration vessel is shown in fig. 76. The stationary and the variable glass electrode are introduced through the top using a two-hole rubber stopper. Two large ports on opposite sides admit a stirrer and the tip of a microburette (c.f. III-12). Both are connected through rubber caps which prevent entrance of air. Another small side arm serves to admit a stream of CO₂-free nitrogen which is passed over the protein solution during the titration in order to keep out atmospheric CO₂. The gas is freed from CO₂ by passage through a soda lime tube. Another small side arm serves as a vent for escaping N₂. With an immersion-type glass-electrode calomel-cell assembly titration may be conducted using an ordinary pH-meter such as the Beckman or Coleman.

Standard N/10 hydrochloric acid for titration may be prepared by dilution of a weighed amount of constant boiling acid to a precisely measured volume.

N/10 KOH is prepared by dilution with CO₂-free water from a saturated solution of KOH (12) (cf. III-16). Traces of carbonate

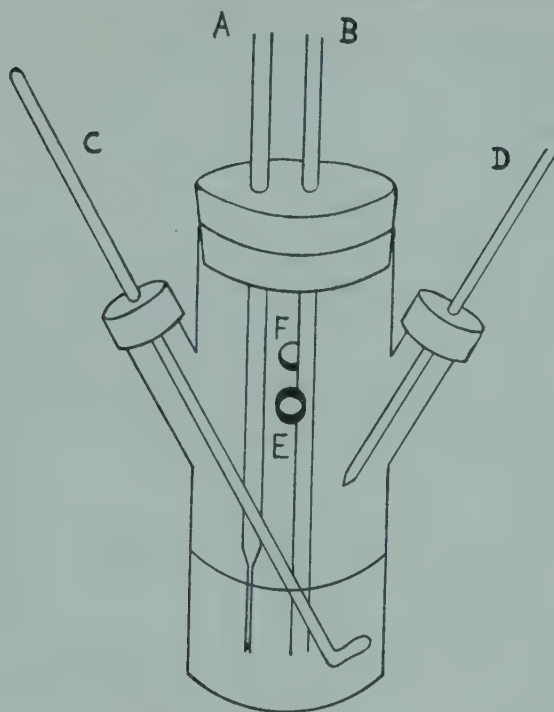


FIG. 76. TITRATION VESSEL. (A) Glass electrode filled with phosphate buffer of about pH 6. (B) Glass electrode into which the protein solution is drawn after addition of each portion of acid or alkali. (C) Stirring rod. (D) Tip of microburette. (E) Small side arm in front to be left open (F) Small side arm in rear for introducing a stream of CO_2 -free nitrogen gas.

remaining in the solution may be precipitated by addition of BaCl_2 solution. Before use in a titration, the N/10 alkali should be tested with BaCl_2 for absence of carbonate.

The protein solution is titrated by addition of 0.02 ml. portions of acid or alkali with measurement of e.m.f. at each step. After completion of the experiment, a portion of the protein solution is analyzed for carbonate. If appreciable amounts of CO_2 are found, it is better to repeat the titration rather than to attempt to correct for the effect of CO_2 .

From the initial pH and the e.m.f. difference after each addition of acid or alkali, the pH at each step is calculated, using the relation:

$$\text{pH} = \frac{5040.2 \cdot \Delta E}{T}$$

in which E represents the e.m.f. and T the absolute temperature.

A 0.15 M solution of KCl is titrated in the same manner to obtain free H^+ or OH^- as a function of pH. Acid or alkali bound at each value of pH is obtained by subtracting the free hydrogen or

hydroxyl ion concentration from the total acid or base added. It is customary to express the amount of acid or alkali bound as equivalents per gram or per mol. of protein and to plot these values against pH.

Formol titration: See references (14-16).

TABLE 1
Characteristic Acidity Constants and Heats of Ionization of Acidic and Basic Groups Found in Proteins

Group	pK (25°C)	H (cal/mol)
Carboxyl (α)	3.0— 3.2	± 1500
Carboxyl (aspartyl)	3.0— 4.7	± 1500
Carboxyl (glutamyl)	ca. 4.4	± 1500
Phenolic-hydroxyl (tyrosine)	9.8—10.4	+6000
Sulfhydryl (cysteine)	9.1—10.8	
Imidazolium (histidine)	5.6— 7.0	+6900 to +7500
Ammonium (α).....	7.6— 8.4	+10,000 to +13,000
Ammonium (α , cystine)	6.5— 8.5	
Ammonium (ϵ , lysine)	9.4—10.6	+10,000 to +12,000
Guanidinium (arginine)	11.6—12.6	+12,000 to +13,000

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CHAPTER 32

OPTICAL ROTATION

Many of the substances studied by the immunochemist, especially the carbohydrates and proteins, possess the power of rotating the plane of vibration of polarized light, by virtue of the centers of asymmetry in their molecules. Measurement of the direction and magnitude of this rotation offers a very useful method of characterizing and identifying optically active substances. In certain instances, the measurement of optical rotation may be used for quantitative estimation of the amount of the substance present.

The measurement of the degree of optical rotation is carried out with a polarimeter or polariscope. Complete descriptions of various instruments may be found in (1, 2). A polarimeter consists of two Nicol prisms placed with their axes on a straight line as shown in fig. 77. Light from the source (S) entering the first prism N (called the polarizer), which permits only light vibrating in a single plane to pass through, emerges as plane polarized light and then enters the second Nicol prism, N (analyzer) to which a circular scale is attached. Depending on the position of the analyzer, the light will either pass through or be partially or totally blocked. If the position in which no light passes through the analyzer is considered as 0° on the scale and a non-optically active liquid or solution or solid (T) is placed between the polarizer and the analyzer (see fig. 77) no light

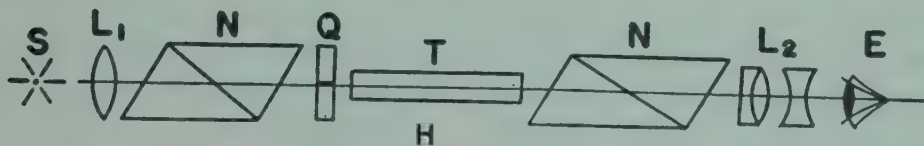


FIG. 77. OPTICAL DIAGRAM OF A POLARIMETER.

From (1). *Polarimetry, Saccharimetry, and the Sugars*. Bates, F. J. and Associates: Natl. Bureau of Standards Circular C440, 1942

will pass through the analyzer if it remains set at 0° . However, if a solution of an optically active substance, such as sugar, is placed between the polarizer and the analyzer, it will be observed that the 0° setting of the analyzer will no longer completely block the passage of light and that it will be necessary to rotate the analyzer to restore to the point of maximum darkness. The extent of this rota-

tion in angular degrees is a measure of the optical rotary power of the solution.

Since location of the point of maximum darkness is difficult (1, 2), modern polarimeters have an optical arrangement which divides the light field into 2 halves and measurements are made by adjusting the setting of the analyzer until both halves are equally illuminated. The difference in analyzer settings obtained with the polariscope tube filled with solution and that obtained with the same tube empty gives the observed rotation.

The magnitude of the rotation is proportional to the concentration of substance in the solution, the length of the column of solution through which the light passes and also depends upon the wavelength of light used, and to a slight extent on the temperature of the solution. For standardizing measurements, a constant called the specific rotation, $[\alpha]_D^t$, characteristic for a given substance has been introduced. It may be defined as the rotation in angular degrees, produced in monochromatic plane polarized light of wave length 5893Å corresponding to the D line of sodium, when it passes through a column of solution 1 decimeter long containing 1 gm. of solute per ml. of solution at 20° C. For many measurements, green light (Hg) at 5461Å is used. From measurements of the rotation produced by any solution of known concentration in a tube of known length, the specific rotation may be calculated, using the following formula:

$$[\alpha]_D^{20} = \frac{\alpha v}{l \cdot w}$$

where α is the observed rotation in degrees, w is the weight of solute (gms.) in v ml. of solution and l is the length of the column of liquid in decimeters (1 decimeter = 10 cm.).

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CHAPTER 33

DIALYSIS AND ULTRAFILTRATION

Since cellophane sausage casing became available commercially*, the use of collodion membranes has been largely abandoned except for ultrafiltration of large volumes at comparatively high pressures as described below or in the Simms' apparatus (1), and except in cases in which the porosity requirements are not met by a cellophane membrane.

Cellophane sausage tubing is marketed in flat, continuous rolls. When it is not necessary to keep the cellophane tube dry, the wet tubing may be opened by rubbing gently between two fingers. If it is to be kept dry the collapsed tubing may be opened by inserting a 26 gauge hypodermic needle between the collapsed walls near the fold. When separated, a suitable length of tubing may now be opened by blowing air into it. To avoid repetition of this process, a slightly greater length than required is blown open and the needed amount cut off, leaving a small length still separated.

The tubing, preferably when wet, may be closed by tying a double knot at one end with dental floss or thread or by knotting the tubing. Fill the tube with water to test for leaks. If no holes are found, introduce the solution to be dialyzed by means of a pipette or funnel. Then tie a double knot at the upper end and immerse the entire bag in the fluid.

For pressure dialysis, it is necessary to insert a one-hole rubber stopper at one end of the cellophane tube. Open up a section of tubing without wetting, as described above, and insert a tapered ground glass stopper of about the same diameter as the rubber stopper to be inserted later. Rotate the glass stopper inside the end of the cellophane tubing to distend it slightly. Then withdraw the glass stopper while holding the tubing gently to retain its circular shape. The rubber stopper may now be inserted without difficulty. The tubing is secured to the stopper with several rubber bands. The other end of the tube may be closed by tying a knot.

Procedure for dialysis: Substances which are subject to decomposition by microorganisms are dialyzed in the refrigerator. A few drops of chloroform (not for proteins) or toluene are added

*Available from the Visking Corp., New York City.

to the fluid both inside and outside the dialysis bag. The outside fluid is replaced once or twice daily and with each change the contents of the bag are mixed and a few drops of toluene are added to the outside fluid to prevent putrefaction.

If the amount of outside fluid is about ten times as large as the quantity inside the bag, four to six changes of dialyzate usually suffice, unless very large amounts of salt or difficultly diffusible ions are present initially. If the volume of dialyzate is smaller, more changes may be required. Whenever possible completeness of dialysis should be verified experimentally by measurement of physical properties like conductance or refractive index, or by chemical tests.

Changes in volume almost invariably occur during dialysis. When a protein solution containing much salt is dialyzed against a weak salt solution or against water, the volume of fluid inside the bag increases considerably. Conversely, when a protein solution containing little or no electrolyte is dialyzed against a more concentrated salt solution, its volume decreases. If a large increase of volume is expected, sufficient empty space (air-free) should be left in the cellophane bag to avoid the danger of bursting.

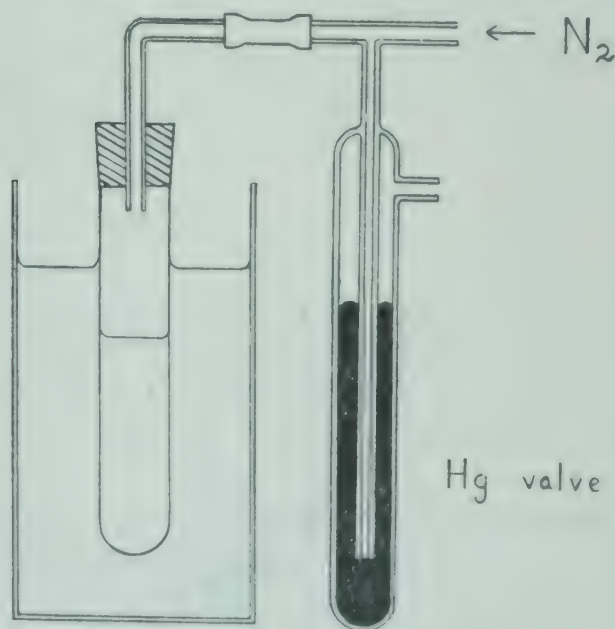


FIG. 78. APPARATUS FOR POSITIVE PRESSURE DIALYSIS. Pressure is supplied from a tank of nitrogen gas. The safety valve contains a column of mercury, the height of which is varied to give the desired pressure. Nitrogen gas should be admitted slowly at first. When nitrogen begins to pass through, the mercury valve will regulate the gas flow to a slow trickle as indicated by an occasional bubble emerging from the safety valve.

Pressure dialysis: 1) Positive pressure. Insert a one-hole rubber stopper into a cellophane tube. Secure a tight fit with several rubber bands. Insert a glass tube into the stopper and connect to a tank of nitrogen gas with a safety valve which limits the pressure to a maximum of 15 cm. Hg. The construction of the entire apparatus, including the valve, is shown in fig. 78.

2) Negative pressure. Prepare a cellophane bag with rubber stopper as described above and insert a long-stem funnel. As shown in fig. 79, the funnel bears a second rubber stopper which serves to hold the bag and funnel in a glass cylinder. This stopper must make an air-tight joint. Suction is applied to the outside fluid by means of the hydrostatic pressure exerted by the column of fluid in the rubber tube leading from the dialyzate to a receptacle placed 3 or 4 feet below the dialysis apparatus. Although concentration proceeds more slowly in this apparatus than in the positive pressure device, it is easier to introduce additional protein solution for concentration through the funnel. If a three hole rubber stopper is used, the dialyzate may be changed continuously by a connection to a fluid reservoir. The rate of flow of fluid must not exceed the capacity of the siphon or the dialysis bag will collapse.

Rotating dialysis: The process of dialysis is accelerated by the arrangement used by McMeekin (2), in which the dialysis bag is rotated, (fig. 80).

Continuous flow exchange of dialyzate: Even greater speed of dialysis can be achieved with the aid of a device which continuously admits fresh dialyzate fluid. An apparatus of this type is shown in fig. 81. The use of tap water for continuous flow dialysis is not advised with proteins since the chlorine usually present may react.

Ultrafiltration for the concentration of proteins and polysaccharides. Proteins and polysaccharides may be concentrated by pressure dialysis using cellophane tubing, but this technique is not practical for processing more than a few hundred cubic centimeters of fluid. Much larger volumes may be manipulated by ultrafiltration using a collodion membrane supported on an alundum cylinder. The method described here has been used successfully on as much as fifty liters of bacterial broth in the preparation of tuberculin (3) and

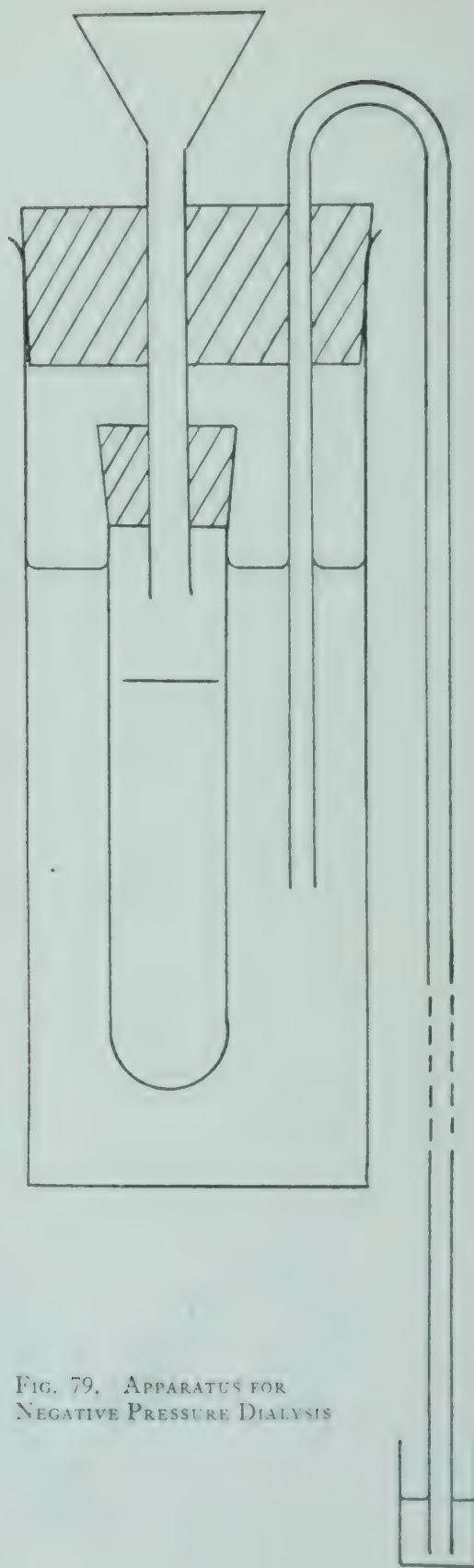
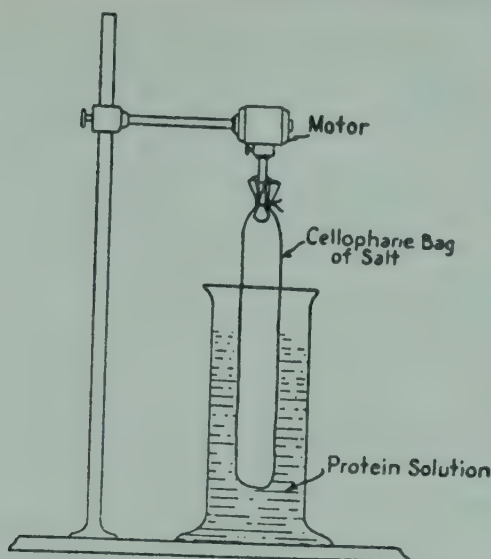


FIG. 79. APPARATUS FOR
NEGATIVE PRESSURE DIALYSIS



Courtesy of American Chemical Society

FIG. 80. Diagram of the apparatus used for adding reagents to protein solutions. *From (2).*

of type-specific pneumococcal polysaccharides (IV-51). It has also been employed in the purification of azo-dye proteins (IV-44).

Apparatus and materials: Alundum cylinder: Arthur H. Thomas No. 5154 C. Parlodion (Pyroxylin purified): Mallinckrodt.

Procedure: For the ultrafiltration of pneumococcus broth in the preparation of type-specific polysaccharide, a 4 per cent solution of parlodion in glacial acetic acid is usually satisfactory. Substances of lower molecular weight like pneumococcus "C" substance and meningococcal polysaccharide may not be completely retained by membranes made from a 4 per cent solution, in which case a higher concentration of parlodion may be required.

Solution of the collodion may require several days. When complete, the solution is transferred to a tall glass cylinder or graduate of somewhat greater diameter than the filter candle. If air bubbles are present, the collodion solution is allowed to stand undisturbed for a few hours, so that even tiny air bubbles may escape. Caution in handling glacial acetic acid is advisable to avoid contact with the skin.

The alundum candle is cleaned by incineration in a muffle furnace. Before use, it is washed with water under suction or pressure, and thoroughly dried. A one-hole rubber stopper holding a glass tube about 20 cm. long is inserted tightly, and with the projecting portion of the glass tube as a handle, the candle is slowly dipped into the collodion solution until it is completely immersed. It is then lifted

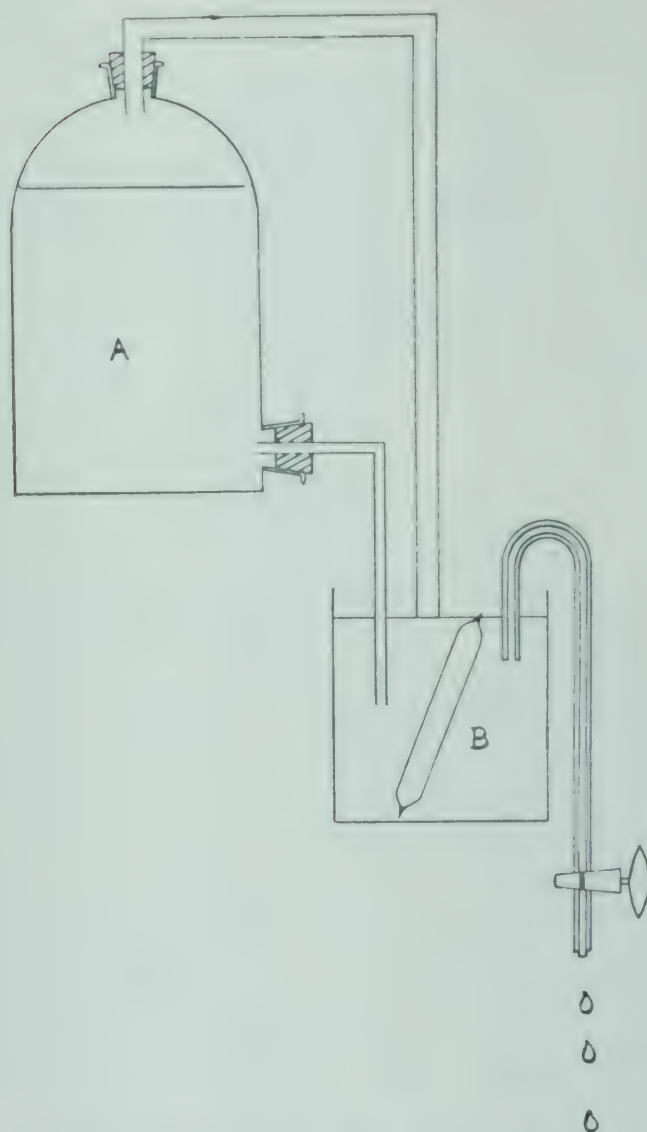


FIG. 81. APPARATUS FOR CONTINUOUS FLOW DIALYSIS. (A) Reservoir. (B) Beaker or jar with dialysis bag. Outflow adjusted to a slow trickle.

out and held in a vertical position to drain. When the excess collodion has dripped off, the candle is carefully transferred to a jar of cold water, taking care not to touch the sides of the jar. Since collodion membranes are rather fragile, coated candles should be treated gently, taking special care to avoid contact of the membrane with other objects.

After the candle has been rinsed in numerous changes of water during several hours, gentle suction is applied and water is drawn through the membrane until the acetic acid is completely removed (test acidity of the filtrate). The candle is then ready for use.

Before ultrafiltration the polysaccharide or protein solution is centrifuged until all traces of turbidity have been removed. If this proves difficult, add celite and filter through paper. In addition, the fluid may be passed through an uncoated alundum candle for clarification. If this candle becomes clogged during the preliminary filtration, it may be cleaned by forcing water through under pressure in the reverse direction. Careful removal of fine particles in this manner serves to avoid premature clogging of the collodion membrane. When a speedy filtration of large volumes is desired, several candles may be suspended in a common vessel.

The suction is furnished by a water pump or an intermittent type vacuum pump. An arrangement of this type permits contin-

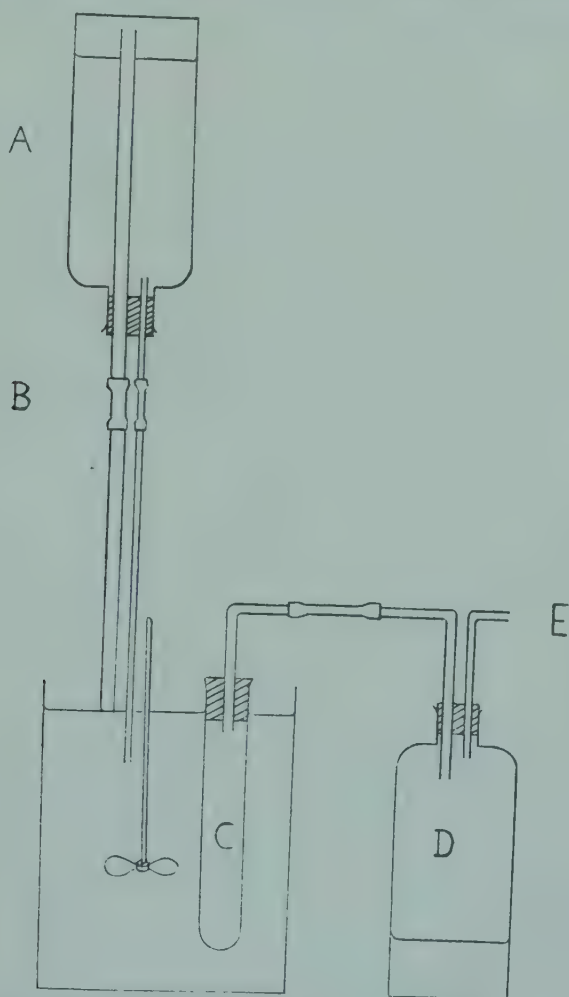


FIG. 82. APPARATUS FOR ULTRAFILTRATION. (A) Reservoir. (B) Place clamp here to stop flow during refilling of reservoir. (C) Alundum candle coated with collodion. (D) Bottle for ultrafiltrate. (E) Outlet to suction pump.

uous operation for several weeks. Fully automatic operation may be achieved by feeding the polysaccharide or protein solution from a large reservoir equipped with a flow control which serves to maintain a constant level in the filtration vessel. The entire apparatus, including the automatic fluid feed is shown in fig. 82. Continuous stirring serves to accelerate filtration.

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CHAPTER 34

DRYING FROM THE FROZEN STATE

The type of apparatus described here has been used successfully on a laboratory scale for the preservation of sera, certain antigens, complement, enzymes, viruses, and other labile biological materials in dry form. Its construction is simple and inexpensive since the only special piece of equipment required is the condenser. The process involves rapid freezing in a dry ice-alcohol cooling bath followed by sublimation under high vacuum. Once the apparatus has been evacuated, the contents of the drying vessel are kept frozen solely by the rapid sublimation of water vapor from the surface of the material.

Apart from making it possible to dry labile substances without deterioration, the process, called lyophilization by Flosdorf and Mudd (1), yields dry products which dissolve completely and with great rapidity, due in part to the porous structure of the dry product.

For drying fluids in bulk, the apparatus is set up as shown in fig. 83. As a general rule, the drying vessel should have a capacity of at least three times the volume of fluid to be desiccated. When a series of small tubes is to be dried a manifold (fig. 83E) is attached to the condenser. Since the speed of drying depends on the rate of diffusion of water vapor to the condenser, all connections should be straight, of large diameter, and minimal length, wherever possible. The drying process may also be hastened by passing a current of air over the vessel to be dried, but care should be taken to avoid melting.

Procedure: The Dewar flask is filled to about one-third its capacity with alcohol or methyl or ethyl cellosolve. The condenser trap is introduced and lumps of dry ice are added, slowly at first, to prevent bubbling over of alcohol. When the alcohol becomes viscous, powdered dry ice is introduced up to capacity. Meanwhile, the drying flask, with its contents, is rotated at an angle in a dry ice-alcohol freezing bath so that the material is frozen as a thin solid shell. Chilling is continued for a few minutes after freezing in order to cool the material sufficiently to prevent melting during the time taken for connecting the drying flask to the condenser and for evacu-

ation. Once a high vacuum has been established the heat loss due to sublimation automatically keeps the material frozen until it is dry, so that cooling is not necessary, and is even undesirable. After about 15 minutes, frost usually appears on the outside of the vessel to be dried, indicating that the apparatus is operating properly. Further attention is not generally required until about 15 to 20 hours later, when the dry ice may have to be replenished.

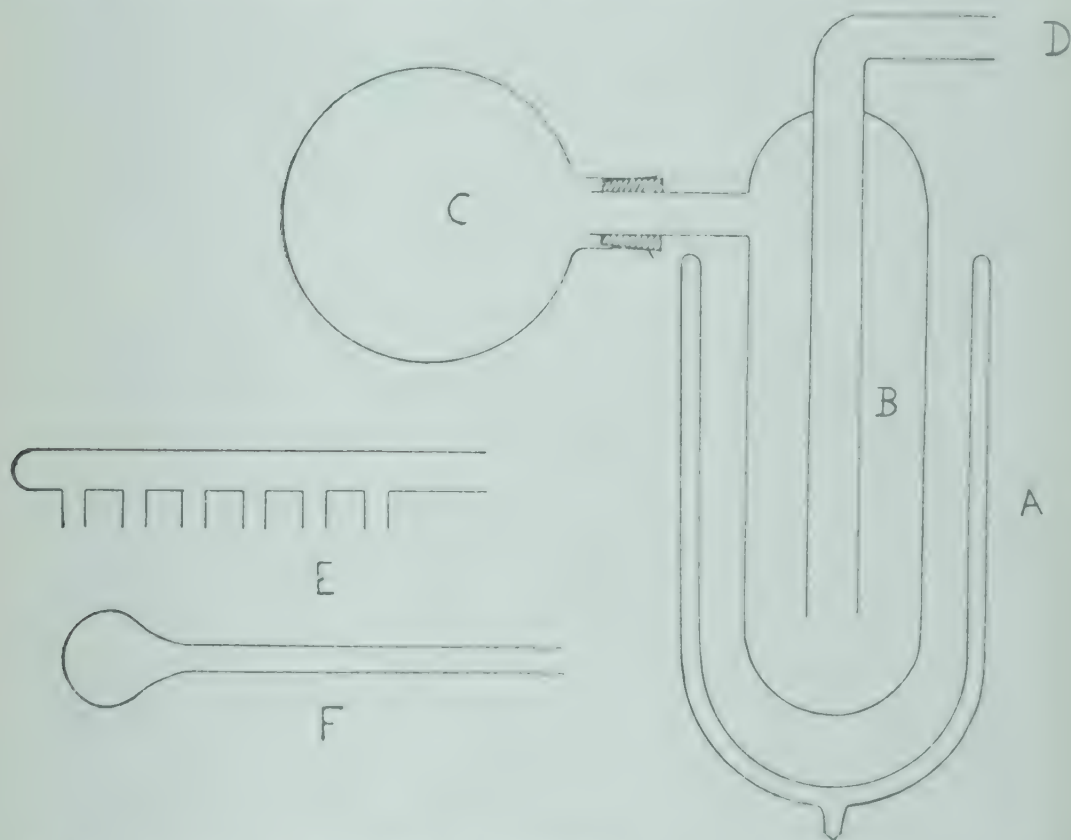


FIG. 83. *Apparatus for Drying from the Frozen State.* (A) Dewar flask. (B) Trap for water vapor. (C) Flask containing material to be dried. (D) Outlet to high-vacuum pump. (E) Manifold for drying small samples in test tubes or ampoules. (F) Pear-shaped ampoule.

As dehydration approaches completion, the frost on the drying vessel disappears and the temperature of the material slowly rises to that of the room. At this point, the material is practically dry, but to remove the last traces of moisture an additional 2 to 3 hours of drying should be allowed.

If it is desired to store the dry product under vacuum, the connection between the drying vessel and the condenser is made with a short length of glass tubing of 5 to 8 mm. diameter which can be

sealed off with a cross-fire torch while the apparatus is under vacuum. When small amounts of fluid (1 to 3 ml.) are to be dried, pear-shaped ampoules (fig. 83F), connected to the manifold (fig. 83E) by means of rubber tubing are used. When the contents are dry, the ampoule is sealed about half-way between the bulb and the rubber tubing.

A small lyophil apparatus does not require a McLeod manometric gauge but a closed-end mercury manometer is useful. A satisfactory vacuum is also indicated by the high pitch of the pump. To test the evacuated apparatus for leaks, the pump is then shut off and the manometer observed for about a minute. If the pressure rises during this period, the connections are not leak-proof and should be checked. The source of leakage may be located with the aid of a high-frequency electrical discharge coil.* The use of thick-walled, flexible rubber tubing of good quality helps to minimize leakage. For efficient operation the pressure should be 50 microns or less, a level easily attained with a high vacuum pump.

An all-glass lyophil apparatus has been described by Campbell and Pressman (2). This device has the advantage that no Dewar flask is needed since the dry ice is kept in the central well of the condenser but requires frequent replenishment of the dry ice.

For work on a large-scale the cryochem apparatus (3) in which the sublimed water is taken up by a chemical drying agent, (e.g., CaSO_4) is said to be more economical. Both lyophil and cryochem machines as well as accessory equipment are available commercially from the F. J. Stokes Co., Philadelphia, Pa.

Recently Strumia and McGraw have described an apparatus for drying plasma in which Freon 12 is used as refrigerant both for prefreezing and condensation (4). Although the cost of construction is high, this apparatus is said to operate at very low cost and appears well adapted for routine hospital use.

The choice of drying vessel depends on the amount of fluid and its intended use. Materials which are to be stored for a long time are best sealed in glass ampoules. If sterility is required, the technique recommended by Appleby (5) is useful. When the period of storage does not exceed a few months, rubber-stoppered vials such as those described by Flosdorf and Mudd (1) are satisfactory. These permit the introduction of water sterilely through a thin membrane

* Coil No. 118-R, manufactured by Eisler Engineering Corp., 736 S. 13th St., Newark, N. J.

section of the rubber stopper. It is, however, advisable to cover the rubber surface with cement.**

For drying a large volume of solution without aseptic precautions, a one or two liter capacity pyrex flask with a short and wide neck may be attached directly to the condenser.

For economy in the use of dry ice as small a condenser trap as possible should be employed. A 1-quart Dewar flask can accommodate a trap 4 cm. in diameter and 30 cm. long, which can hold about 30 to 40 ml. of ice. The inlet and outlet tubes of this trap should be about 16-17 mm. in diameter. For drying up to about 300 ml. of fluid, a trap 10-12 cm. in diameter and 30 cm. long is convenient. The inlet and outlet tubes should be about 22 mm. in diameter. This trap requires a Dewar flask of 1 gallon capacity.

** Flosdorf and Mudd (1) recommend "Dapar Liquid Sealing Cement," made by Rapad Products Co., 8024 Jackson Street, Philadelphia.

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CHAPTER 35

EXTRACTION OF ANTIGENS FROM MICROORGANISMS

While certain bacterial antigens like diphtheria toxin (IV-49), streptolysin and the pneumococcal capsular polysaccharides (IV-51), pass into the culture medium during growth or on autolysis and may be separated by chemical fractionation of the culture fluid, the usual procedure for obtaining antigens from microorganisms is by extraction of washed, intact or disintegrated cells with appropriate solvents. Some antigens can be extracted with water, saline or neutral buffers, while others require acid or alkaline media or enzymatic digestion. Certain organic solvents have also been used. In some cases it is possible to extract the washed, intact organisms, but in others it is necessary first to disintegrate the cells mechanically by grinding with sand in a mortar or in a ball mill, by alternate freezing and thawing, or by exposure to sonic waves. In many instances, it has been found advantageous to extract lipids with alcohol-ether, acetone, or chloroform, before applying aqueous solvents. In using any or all of these chemical and mechanical procedures, the possibility of degradation as a result of the treatment must be considered.

Boor and Miller (1) extracted nucleoproteins from *Neisseria* and from pneumococci using cold water and enough NaOH to keep the pH at 7.6-7.8. With staphylococci and streptococci, satisfactory extraction required 0.01 *N* NaOH (1). Wong and T'ung (2) extracted type-specific protein from defatted and partially dried diphtheria bacilli using 0.05 *N* NaOH. Treatment with 2 per cent phenol solution at 0°C. was used by Miles and Pirie (3) to extract soluble, specific substance from *Brucella melitensis*. Furth and Landsteiner (4) dissolved various species of *Salmonella* in sodium hypochlorite and precipitated carbohydrate possessing O-antigen specificity by addition of alcohol to the solution. Type-specific M-substance from group A hemolytic streptococci has been extracted at 56°C. from dried, whole cells with 0.05 *N* HCl containing 2 per cent NaCl (5) or at 37° C. with 0.10 *N* HCl (5a). One-quarter *N* trichloroacetic acid has been employed to extract Boivin type antigens from *Salmonella* (IV-50).

Morgan (6) isolated the specific polysaccharide of *B. dysenteriae*

Shiga by extraction of washed and acetone-dried bacteria with diethylene-glycol. Henderson and Morgan (7) extracted acetone treated, dried typhoid bacilli with ethyleneglycol. Boor and Miller (1) attempted the use of surface active agents like sodium oleate, sodium ricinoleate and sodium taurocholate, but these dissolved pneumococci only. Avery and Heidelberger (8) prepared crude pneumococcal nucleoprotein by solution of the organism in bile followed by isoelectric precipitation with acetic acid. Concentrated phenol (88-95 per cent) has been found to be a good solvent for most proteins, while many polysaccharides like those from virulent pneumococci are insoluble. Those polysaccharides which are soluble may often be precipitated from phenol solution by addition of alcohol or glacial acetic acid. Palmer and Gerlough (9) prepared polysaccharide antigens from typhoid bacilli using phenol for the removal of protein. Blood group A substance (IV-53) can also be precipitated from phenol extracts of hog gastric mucin by addition of alcohol to 10 per cent by volume. Phenol may be removed from extracts by dialysis.

The "C" polysaccharide of streptococci was extracted by Zittle and Harris (10) using Fuller's (11) formamide method. The acid-extracted streptococci were dried with petroleum ether and treated with formamide for 20 min. at 150° C. The use of urea solutions to extract antigens from bacteria of the *Salmonella* group has been described by Walker (12).

For extraction of non-protein antigens bacteria may be digested with trypsin. Raistrick and Topley (13) used a preparation of this enzyme which also contained erepsin to disintegrate acetone-dried *B. aertrycke*.

In many cases it is, however, necessary to disintegrate cells by physical means before extraction with a solvent. Lancefield (14) ground dried streptococci with fine sand in a ball mill (cf. 15), and then extracted the nucleoprotein with 0.01 *N* NaOH. An improved procedure by Heidelberger and collaborators (16, 17, 18) was designed to avoid alterations in serological properties by successive extraction with a series of buffers of increasing alkalinity. Since this method appears to be of general applicability, it is given in detail (16).

About 50 to 60 l. of a 3-day old broth culture of group A hemolytic streptococci were run through a Sharples centrifuge. The organ-

isms were collected from the bowl, suspended in about 400 ml. of saline and centrifuged in the cold. (All subsequent treatments were in the cold). The washed cells were suspended in about 400 ml. of acetone, stirred frequently during 2 hours, centrifuged, taken up again in the same quantity of acetone and allowed to stand overnight. After centrifugation, the cells were taken up in 400 ml. of ether which had been freed from alcohol, peroxides, and aldehydes by washing with water, drying with calcium chloride and storing in the dark over calcium chloride and sodium hydroxide pellets. After frequent stirring during several hours the mixture was centrifuged and the process repeated with a new lot of purified ether. After a third treatment with ether, which lasted overnight, the mixture was centrifuged and the organisms were dried *in vacuo*.

The defatted cells, weighing about 10 gms. of which about 10 per cent was ash, were ground in a ball mill (preferably with stone balls) until intact cells could no longer be observed in a smear. The ground material was then rotated in the ball mill with a little 0.2 *N* acetate buffer at pH 4 for 10 minutes, transferred into 5 l. of the same buffer solution and stirred mechanically for 5 to 6 hours in the cold. The mixture was run through a Sharples centrifuge and the "C" polysaccharide was isolated from the extract.

The residue was ground in a mortar with chilled *M/15* phosphate buffer at pH 6.5, added to 4 l. of the same solution and stirred mechanically for 6 hours. After centrifugation yielding a fraction D in the supernatant, the residue was treated successively with solvents at pH 8.4, 9 and 11, which resulted in the extraction of fractions E, F and G. Fraction D was shown to be an alkali-labile nucleoprotein since nucleic acid was split off by treatment with NaOH. In later studies on a type 1 strain of group A hemolytic streptococci (19) preliminary drying and removal of lipid with acetone and ether was omitted and the washed cells were ground with appropriate buffer solutions in a stone-ball mill partially immersed in ice-water. Grinding in a ball mill has also been used to extract proteins from the timothy grass bacillus (20, 21) and from bovine and avian (21), and human (22, 23) strains of the tubercle bacillus. Boor and Miller (1) disintegrated gonococci and meningococci with the aid of a ball mill. Verwey (24) employed it for grinding staphylococci. A bacterial grinding mill of special design

has been described by Booth and Green (25). It was used by Hudleson to disintegrate *Brucellae* (26).

The separation of polysaccharides from large quantities of ground bacteria may be illustrated by the procedure of Heidelberger and collaborators (27, 28) for extracting tubercle bacilli. The dried, defatted and ground bacilli were triturated in a mortar with 3.5 per cent acetic acid and placed in a percolator provided with a filter plate covered with filter paper, asbestos and a layer of purified sand. The mass was then percolated under slight suction with 0.35 per cent acetic acid containing 0.5 per cent phenol. Polysaccharides were isolated from about 115 l. of extract accumulated during 7 months' percolation.

Disintegration by alternate freezing and thawing was applied to *C. paratuberculosis* by Gunnison (29). The washed organisms were suspended in water, 0.9 per cent NaCl, or in an alkaline solution of pH 8, and the suspensions were frozen in a mixture of crushed ice and salt followed by rapid thawing through immersion in warm water. It was necessary to repeat this process from 15 to 30 times before the organisms showed microscopic evidence of disintegration. Boor and Miller (1) subjected gonococci and meningococci to four alternate freezing and thawing operations but observed only little disintegration on microscopic examination.

Disintegration of bacteria by sonic vibrations (30) of 8.9 kc. generated by magnetostriction has been used with a number of organisms including *E. typhi*, *Streptococcus hemolyticus* (30), *H. pertussis* (31, 32), pneumococci (33) and streptococci (34). Sonic treatment is, however, not harmless since Flosdorf and Chambers showed that egg albumin was denatured by sonic vibrations (35). An apparatus to produce supersonic waves has been described recently by Smith, Stumpf and Green (36). The chief advantages of this device lie in its rapidity of action, its ability to handle small amounts of material and the fact that the bacteria can be treated in any thin-walled, flatbottomed glass vessel. It is thus easy to maintain sterility. Certain bacteria like *Micrococcus lysodeikticus* and *Sarcina lutea*, could, however, not be disintegrated with the power available (37). In the supersonic apparatus a 1-inch quartz crystal immersed in a cooled oil-bath emits supersonic waves of 600 kc. driven by a radio-frequency generator rated at 2000 volts with an output of 600 watts. The flask containing the bacterial suspension is held in the oil-bath

a few millimeters above the quartz crystal the optimum distance being found by experiment. Results obtained with various microorganisms are given in table 1. The time of exposure usually employed is about 15-20 min. It has been found that more efficient disintegration is obtained if the temperature is allowed to rise to about 40-45° C. With labile substances this may not be desirable, however.

TABLE 1

Effect of Supersonic Waves on Various Microorganisms

Easily disintegrated	Refractory
Hemophilus influenzae	Sarcina lutea
Salmonella typhimurium	Saccharomycetes cerevisiae
Lactobacillus casei	Micrococcus lysodeikticus
Lactobacillus delbrueckii	Acetobacter suboxydans
Proteus vulgaris	
Proteus morganii	
Escherichia coli	
Pseudomonas pyocyaneus	
Staphylococcus aureus	
Erythrocytes	
Brucella abortus	
Clostridium welchii	

From (37)

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CHAPTER 36

CRYSTALLINE HEN'S EGG ALBUMIN

Crystalline egg albumin is a relatively homogeneous protein as indicated by constancy in composition and solubility (1, 2) and quantitative immunochemical studies (3) have shown that it behaves as a single antigen in its reaction with rabbit antisera. Accordingly, it has been widely used in immunochemical investigations (cf. I, 2, 5, 6; II, 7, 9, 10; IV 44, 46, 47) and for studies on the mechanism of immune reactions (3). Recently, it has been established that crystalline egg albumin actually may show two electrophoretic components in the pH range 5 to 10 (4), and that the more slowly migrating component increases with the age of the preparation (5), at the expense of the faster component and is usually the only component present after 1 year. Nothing is known of the effect of this transformation on the immunochemical properties of egg albumin.

Since the method of crystallization with ammonium sulfate (6) is more difficult, the procedure of Kelwick and Cannan(7) is given.

Preparation (7): A solution of Na_2SO_4 is prepared by dissolving 400 gm. of the anhydrous salt in 1 liter of warm water. This solution which contains 36.7 gm. of salt in 100 ml. must be kept at a temperature above 30 degrees to prevent crystallization. The whites of fresh eggs are collected and the membranes broken up with an egg beater diluted with an equal volume of water and a volume of the salt solution equal to that of the diluted egg white is added slowly with stirring. The mixture is stirred for some time and after 1 to 2 hours the precipitate is removed by filtration or by centrifugation. A solution of 0.2 N H_2SO_4 is slowly added to the filtrate which is stirred mechanically. Addition of acid is continued until the pH is about 4.6-4.8. A test on a few drops of the mixture with bromocresol green is sufficiently accurate. It is unusual for any permanent precipitate of protein to separate during addition of acid but, if this should occur, the precipitate is redissolved by addition of minimal amounts of water. After the desired pH has been attained, stirring is continued and a saturated solution of Na_2SO_4 is added dropwise with stirring from a narrow orifice submerged below the surface of the liquid until a permanent opalescence develops. Addition of Na_2SO_4 solution is stopped but stirring is continued. When crystalliza-

TABLE 1
Properties of Crystalline Hen's Egg Albumin

Method of preparation	Nitrogen content	Carbo- hydrate content	Phosphorus content	Electrophoretic mobility of com- ponents $\times 10^5$ at pH 6.8*	Sedimen- tation constant S_{20}	Diffusion constant $D_{20} \times 10^7$	Molecular wt. (from S_{20} and D_{20})	Frictional coefficient f/f_0	Isoelectric point	Optical rotation $[\alpha]_D$
Ammonium sulfate (6)	15.4 (13)	1.71 † (11)	0.77 (2)	—5.9 to —6.5 (4,5) —4.8 to —5.6 (4,5)	3.54 (8)	7.7 (9)	43,800	1.16	pH 4.55 (10)	—30.5 (6)
Sodium sulfate (7)	15.4 (12)			—5.3 to —5.4 (5) —5.0 (5)						

* Phosphate buffer, ionic strength 0.1

† Determined as mannose.

tion of the protein becomes clearly evident, the mixture is allowed to stand for a day or two. The entire procedure must be conducted in a room not cooler than 20 degrees or it will be impossible to achieve a high enough concentration of salt to effect crystallization of the protein.

The crystals are removed by filtration or by centrifugation and redissolved in a volume of water about equal to one-half the original volume of egg white. Recrystallization is effected by dropwise addition with stirring of a volume of saturated Na_2SO_4 solution equal to the volume of water used to dissolve the precipitate. After two further recrystallizations the packed crystals are either dissolved and dialyzed or converted into a powder by drying in air. The dry product has been shown to retain its ability to crystallize after storage at room temperature for 2 years. Electrophoretic diagrams showing the progressive purification achieved in successive recrystallizations have been published in (4).

An advantage in the use of Na_2SO_4 for crystallization is the elimination of ammonium ion (6) from the product. Dialyzed solutions of egg albumin which have been crystallized from $(\text{NH}_4)_2\text{SO}_4$ retain amounts of NH_4^+ which depend on the pH of the solution. Some properties of crystalline egg albumin are listed in table 1.

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CHAPTER 37

CRYSTALLINE SERUM ALBUMINS

Horse Serum Albumin: Crystalline horse serum albumin has been widely used in immunochemical investigations since it is so readily obtained. Chemical, physical and immunological studies, however, have shown that, as usually isolated, it may not be a pure protein. Goldsworthy and Rudd (1) demonstrated by precipitin tests that even three-times recrystallized albumin prepared according to Adair and Robinson (2) contained as much as two per cent of globulin as impurity. Kabat and Heidelberger (3) showed that this was responsible for the occurrence of a broad zone (3a) in which both antigen and antibody could be detected in the same supernatant fluid in their quantitative precipitin studies. The production of anti-globulin could be avoided by using relatively small amounts of albumin for immunization. Under these conditions the reaction of crystalline albumin and antiserum behaved as a single antigen-antibody system.

Soerensen (4) found that crystalline horse serum albumin was inhomogeneous using phase rule criteria for solubility, and that the more soluble fractions were relatively rich in carbohydrate and phosphorus. He obtained products ranging from 0.02 to 0.47 per cent in carbohydrate content (5).

Using the mother-liquors from repeated crystallizations of the albumin, Hewitt (6, 7) was able to separate a carbohydrate-rich protein (8.6 per cent) which he called seroglycoid. McMeekin (8) succeeded in crystallizing a carbohydrate-rich protein (5.5%) from the mother-liquor after removal of crystalline carbohydrate-free albumin. McMeekin's glycoprotein was electrophoretically and ultracentrifugally homogenous. Its electrophoretic mobility was less than that of carbohydrate-free "crystalalbumin". The crystals were symmetrical hexagonal discs while the carbohydrate-free albumin crystals are rod-shaped.

Following the method of Adair and Robinson (2), Mayer and Heidelberger (9) obtained crystalline albumin essentially free of carbohydrate and phosphorus. McMeekin (10) also prepared carbohydrate-free crystalline albumin by $(\text{NH}_4)_2\text{SO}_4$ fractionation. This product also failed to satisfy the phase rule criteria for the

solubility of a single chemical individual (cf. III, 29) since its solubility was not independent of the amount of protein in the system and did not remain constant in successive equilibrations. A fraction of constant solubility, independent of the amount of protein in the system, was obtained by crystallizing the albumin-sulfate (fig. 84)

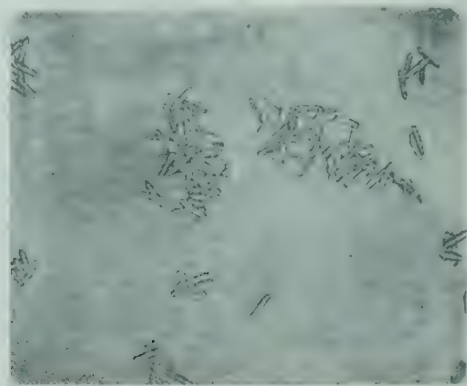


FIG. 84. Microphotograph of serum albumin sulfate crystallized from water. *From (10).*

at pH 4.0 from a concentrated aqueous solution. The albumin-sulfate was electrophoretically homogeneous at pH 7.4 but still showed a small quantity of a second component at pH 4.0. Its nitrogen content by Kjeldahl analysis was 16.8 per cent compared to 16.1 per cent for the unfractionated albumin and 15.8 per cent (5, 9) for the albumin prepared according to Adair and Robinson.

Kekwick (11) prepared crystalline albumin using Na_2SO_4 and separated it into two fractions, A and B, the former of relatively high solubility and high carbohydrate content, and the latter with low solubility and low carbohydrate content. Both fractions sedimented at the same rate in the ultracentrifuge, and had identical diffusion constants (11, 12) and molecular weights. They also migrated at the same rate on electrophoresis at pH values ranging from 3.6 to 7.6 (11, 13).

Rimington and Van den Ende (14) crystallized a fraction from albumin which had been called globoglycoid by Hewitt (15). After four crystallizations they obtained globoglycoid free from carbohydrate. By chemical methods and by anaphylactic tests using the Dale technic (I, 5) they demonstrated that globoglycoid was identical with crystalline albumin.

The procedure of Adair and Robinson (2) is the most widely used for the preparation of crystalline carbohydrate-free albumin:

PREPARATION

Method of Adair and Robinson (2): (Manipulations at room temperature except as noted). One liter of fresh horse serum from a healthy normal animal, is diluted with 1 liter of water. Two liters of saturated ammonium sulfate solution are added slowly with mechanical stirring (1-2 hours). The globulin precipitate is removed by centrifugation and the supernatant is acidified very slowly (3-4 hours) by dropwise addition with mechanical stirring of about 200 ml. of the following mixture: One volume of 0.5 *M* acetic acid + 1 volume saturated ammonium sulfate. Crystallization of serum albumin usually begins during acidification and should be complete after standing overnight at room temperature. The final pH should be about 4.9. Spot tests with brom-cresol-green are sufficiently accurate.

The following day the crystals are separated by centrifugation and washed with an approximately equal volume of a solution prepared by adding 600 ml. of *M* sodium acetate solution and 400 ml. of *M* acetic acid to 1000 ml. of a saturated solution of ammonium sulfate. It is possible to wash the crystals with this fluid two or three times without much loss.

The quantities of reagents used for recrystallization given below are based on 1000 ml. of serum. The crystals are taken up in about 500 ml. of water and 80 ml. of *M* sodium acetate are added to effect solution. After filtration, to remove any insoluble residue, a volume of saturated ammonium sulfate solution equal to the sum of the volumes of the water and the sodium acetate (about 580 ml.) is added. The acidity is then adjusted to pH 4.9 by the addition of 106 ml. of a mixture composed of equal volumes of *M* acetic acid and saturated ammonium sulfate. The only reagent which must be added slowly is the mixture of acetic acid and ammonium sulfate, which should be added to the albumin solution during a period of about 1 hour. The pH is again checked with brom-cresol-green indicator. Crystallization takes place within 2 hours, and may occur sooner if the amounts of water and of ammonium sulfate are reduced, but under these conditions the crystals are very small. The solution must be stirred during the addition of acid and it is also advisable to stir the solution during crystallization.

The yield may be increased by leaving the preparation overnight at room temperature. The crystals are separated from the mother-

liquor by centrifugation, and are redissolved and recrystallized by the same method. As shown by electrophoretic analysis (16), two

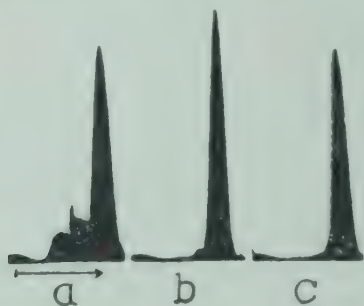


FIG. 85. Electrophoretic pattern of fresh horse serum albumin after (a) initial crystallization, (b) first; and (c) second recrystallizations. *From (16).*

recrystallizations suffice to remove all globulin detectable by this technic (fig. 85), but for immunological purposes 5 recrystallizations are recommended (3).

As in the case of egg albumin (16b) it has also been shown that prolonged storage of crystalline serum albumin solutions even in the cold caused changes which led to the appearance of a second electrophoretic component (16), an observation made earlier by Tiselius (16a).

Preparation of serum albumin sulfate (10): A salt-free solution of purified carbohydrate-free albumin containing 26 g. of protein in 260 ml. was brought cautiously to pH 4.0 by adding 0.1 normal sulfuric acid (approximately 0.00025 mol of acid being required for each gram of protein) and then concentrated in a negative pressure dialyzing apparatus at 4° C. (III, 33). When the protein concentration reached about 25 per cent the solution was brought to room temperature. Crystallization began within an hour and was allowed to continue for four hours. The crystals were removed by centrifugation and washed three times, each time with a volume of water equal to the volume of the precipitate. The supernatant was again concentrated at 4° C. and a second and third crop of crystals removed. Crystallization was continued until no further separation occurred. The first crop of crystals generally amounted to 10 g. of protein, the second to 3 g. and the third to 0.3 g., leaving 12.5 g. of protein dissolved in solution under these conditions. Thus, about one-half of the original serum albumin was converted into water insoluble crystalline material.

Method of Kekwick (11): As a general rule, the serum was worked up in 4 l. batches, all the procedures being carried out in a warm room at 30° C. unless otherwise stated.

The globulins were precipitated by addition of 20 g. Na_2SO_4 per 100 ml. serum and allowed to stand over night before filtration. The albumin filtrate was then acidified with 0.2 *N* H_2SO_4 , the usual precautions being observed (continuous mechanical stirring; delivery of the acid from a small orifice below the surface of the liquid) until an apparent pH of 4.8 to 4.9 was attained. This usually required 230 ml. acid per l. of filtrate. The pH was controlled externally with bromo-cresol green as an indicator, allowance being made for the color of the serum and for the effect of the Na_2SO_4 present.

Crystallization usually commenced as the final amounts of acid were stirred in; then for every 100 ml. of acid added 20 g. of anhydrous Na_2SO_4 were stirred in and crystallization was allowed to come to completion by standing for 24 hours.

After standing, the crystals were filtered off and allowed to drain thoroughly. The filtrate was tested for further crystallization; it was found that either a slight amorphous precipitate formed, or if there were any crystals the amount was negligible.

Separation into fractions A and B: The carefully drained crystals were extracted at 30° C. with a volume of distilled water equal to one-tenth that of the initial serum, the extraction being continued for 16 hours with occasional stirring (Fraction A). The undissolved material, mostly crystalline but showing some amorphous substance was then filtered off, drained and extracted for several days at 2° C. with a quantity of distilled water equal to that used in the first extraction (Fraction B), after which the green amorphous residue was filtered off and discarded.

Recrystallization: Recrystallizations were carried out at 30° C. and conditions were sought such that at each recrystallization between 85 and 95 per cent of the fractions was recovered. For fraction A this was achieved by recrystallization from solutions containing 3.0 gm. protein per 100 ml., the sodium sulfate concentration being brought up to 20 gm. per 100 ml. For fraction B the corresponding conditions were 4.5 gm. protein per 100 ml. and 15 gm. Na_2SO_4 per 100 ml. The pH was carefully maintained at 4.8-4.9 as in the initial crystallization procedure.

After 5 recrystallizations, fraction A contained 14.35 per cent N

and 1.95 per cent carbohydrate, while fraction B had 15.16 per cent N and 0.083 per cent carbohydrate.

HUMAN SERUM ALBUMIN

Human serum albumin was first crystallized by Adair and Taylor (17). A procedure reported by Kendall (18) represents an improvement since it has given good yields from numerous samples of normal human serum. The crystalline albumin obtained by Kendall was associated with about 2 to 3 per cent of free fatty acid that could not be removed without first denaturing the albumin. Fractional recrystallization at $\frac{3}{10}$ and $\frac{1}{2}$ saturation with ammonium sulfate yielded two products, the former highly colored with yellow pigment and containing 1.3 per cent lipid, and the latter almost colorless and containing 0.4 per cent lipid. Solubility measurements (18) indicated that the crystalline human serum albumin was much more homogeneous than the corresponding albumin from horse serum, although the measurements did not show unequivocally that the product contained but a single protein component. The material was found to be homogeneous on electrophoresis (18) in the Tiselius apparatus.

Procedure: 100 ml. of undiluted serum or plasma were chilled in an ice bath and 120 ml. of ammonium sulfate solution saturated at room temperature were slowly added from a dropping funnel, during an hour, with mechanical stirring. After vigorous stirring for another hour the precipitated globulin was filtered off in the cold with suction. The filtration was repeated if necessary to give a clear filtrate. The albumin was then precipitated by dissolving 25 gm. of solid ammonium sulfate in the filtrate (75 per cent saturation.) After standing overnight at room temperature the amorphous precipitated albumin was filtered off with suction. The moist precipitate was dissolved in 25 ml. of distilled water and the volume of the solution measured. The hydrogen ion concentration was adjusted to pH 4.9 with 0.1 N H_2SO_4 in half-saturated ammonium sulfate. The solution was cooled to 15° C. and the ammonium sulfate concentration brought to half-saturation by the addition of the calculated amount of saturated solution. The amount needed was 39 ml. minus one-half the measured volume of the albumin solution. This formula assumes the presence of 5 gm. of albumin and makes allowance for the 75 per cent saturated ammonium sulfate solution in the moist

precipitate. A small precipitate formed which was filtered off through a folded filter in the ice box. The clear filtrate was allowed to stand in the ice box at 4°C . until crystallization occurred. Crystallization was very slow, in some cases a period of 2 weeks elapsed before the appearance of the first crystals. Although seeding with crystals accelerated crystallization, at least 2 weeks more were necessary for maximum yields. With the exception of a small amount of flocculent amorphous material which sometimes separated before crystallization began, the precipitate was entirely crystalline, consisting of elongated dodecahedrons with square cross-section (fig. 86).



FIG. 86. Crystalline human albumin X 75. Polarized light. *From (18).*

The crystals exhibited the property of double refraction of polarized light. They were very fragile, being broken up by vigorous stirring or by the pressure of a cover-glass on a microscope slide. They showed a high temperature coefficient of solubility, going into solution in the supernatant fluid if warmed to room temperature, when they were often replaced by amorphous material. From 40 to 65 per cent of the albumin was obtained in crystalline form. The yield could be increased and a second crop of crystals obtained either by concentrating the supernatant solution by ultrafiltration at 0°C . or by precipitating the remaining albumin at 75 per cent saturation with ammonium sulfate and repeating the crystallization procedure from a smaller volume.

Recrystallization of Human Serum Albumin: The crystals from 100 ml. of serum were dissolved in 10 ml. of water and filtered through an asbestos filter. 10 ml. of saturated ammonium sulfate

solution were added at 15° C. without causing precipitation. The solution was cooled to 4° C. and seeded with crystals. Although crystallization was more rapid than before, at least a week was required to give maximum yields. After four crystallizations the crystals were dissolved in a small volume of H₂O and dialyzed in the cold against distilled water until free from ammonium sulfate. The pH of the dialyzed solution was 4.9.

Several preparations were dried *in vacuo* while frozen. The dried material dissolved completely in water to give clear solutions that crystallized when half saturated with ammonium sulfate. Samples of different preparations, when dried to constant weight *in vacuo* over P₂O₅ at room temperature, had a nitrogen content of 15.7 ± 0.2 per cent by the micro-Kjeldahl method. All preparations were free from carbohydrate when analyzed by the orcinol method of Soerensen and Haugaard (5).

The albumin which had been crystallized four times was still colored yellow. Since the first crystals to separate were more highly colored, it was possible to obtain colorless preparations by fractional crystallization as follows:

8 gm. of albumin which had been crystallized four times from half-saturated ammonium sulfate were dissolved in 50 ml. of H₂O, chilled to 10° C., and treated with 30 ml. of saturated ammonium sulfate. The clear solution was seeded with crystals and allowed to stand at 0° C. Crystallization proceeded rapidly, forming crystals indistinguishable from those obtained from half-saturated ammonium sulfate. After 3 days the crystals were centrifuged compactly in the cold and redissolved in 25 ml. of water. Upon the addition of 15 ml. of saturated ammonium sulfate solution crystallization took place as before. After two more recrystallizations the crystals were dissolved in a small volume of water and dialyzed against distilled water in the cold until free from ammonium sulfate. The pH of the dialyzed solution was 4.9. The yield was 5.2 gm. (Fraction I).

The supernatant solutions from these crystallizations were mixed and ammonium sulfate added to half saturation. Crystallization took place slowly after the solution had been seeded with crystals, 3 weeks at 0° C. being required to obtain a maximum yield. After three more crystallizations from half saturated ammonium sulfate this fraction was dialyzed free from salts. The yield, Fraction II, was

1.3 gm. Fraction I was still highly colored with the yellow pigment, while Fraction II was almost colorless.

TABLE 1
Properties of Crystalline Horse and Human Serum Albumins (Sa)

Product	N Content (Kjeldahl)	Carbohydrate content	Optical rotation [α] _D	Sedimen- tation constant S ₂₀	Diffusion constant D ₂₀ × 10 ⁷	Frictional Coeffi- cient	Molecular weight	Specific refraction increment in aqueous sol.
	%	%	degrees	Svedbergs	cm ² /sec	f/fo		
Horse-Sa*	15.6 (2) 15.8 (9)	0.02 to 0.05 (5,9)		4.3 (19)	6.1 (19)	1.27 (19)	68,000 (19)	0.00183 (2)
Horse- Sa A†	14.4 (11)	1.95 (11,12)		4.4 (11)	6.1 (11)	1.27 (13)	70,000 to 72,000 (11,13)	
Horse- Sa B†	15.2 (11)	none to 0.083 (11,12)		4.5 (11)	6.1 (11)		70,000 (11)	
Horse-Sa‡	16.1 (10)	none (10)	-57 (8)	4.1 (8)				
Horse-Sa§	16.8 (10)	none (10)		4.1 (8)				
Horse-Sa**	15.1 (8)	5.5 (8)	-47 (8)	4.1 (8)				
Human- Sa††	15.7 (18)	none						

For data on diffusion and viscosity of serum albumin see (20, 13).

For recent amino acid analyses on carbohydrate-free serum albumin, see (21).

* Crystalline Sa prepared according to the method of Adair and Robinson (2).

† Product obtained by the method of Kekwick (11).

‡ Carbohydrate-free Sa of McMeekin (8).

§ Sa crystallized from water as sulfate (10).

** Crystalline carbohydrate-containing protein of McMeekin (8).

†† Product of Kendall (18).

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CHAPTER 38

GAMMA GLOBULIN

Gamma globulin is a protein of considerable immunochemical interest since in several animal species antibodies are identical in physicochemical properties with this protein (I, 6). Preparations of gamma globulin are frequently used to standardize the Folin-Ciocalteu method for estimating antibodies. Three methods of preparation are given:

Electrophoretic Method: Small amounts of gamma globulin may be prepared electrophoretically from whole serum (I, 2) in the Tiselius electrophoresis apparatus (see III, 25). With the standard size cell, 4-5 ml. of gamma globulin, i.e. about 40-50 per cent of the total γ -globulin in the serum used, may be obtained from 10 ml. of serum in a single run. With the large size cell as much as 20-30 ml. may be obtained from 70-80 ml. of serum. To avoid possible contamination with other serum proteins, it is necessary that the layer containing gamma globulin be separated from the remaining components in the adjacent compartment by means of the flange plates and that the beta globulin component be clearly visible below the plates. To avoid contamination by protein adhering to the walls of the cell, it is desirable, after the apparatus is set up, to start the current and effect a separation of the serum components before starting the compensation mechanism to draw the gamma globulin backward into the upper right compartment. By doing this, the beta globulin boundary can be continuously observed and prevented from entering the compartment from which the gamma globulin solution is to be obtained.

Ammonium Sulfate Fractionation: The water-soluble euglobulin of human serum, as prepared by Kendall (3, 4) and called alpha globulin and subsequently Globulin I, has been shown to be identical with gamma globulin as obtained electrophoretically. Kendall has also shown that it behaves as a single substance immunologically, in its reactions with rabbit antisera to whole globulin, since tests for both antigen and antibody were never obtained on the same precipitin supernatant (cf. II, 8).

The procedure used by Kendall (3) is to precipitate the euglobulin by addition of 50 ml. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution to 100 ml. of undiluted human serum. After centrifugation the precipitated

euglobulin is dissolved in 50 ml. of water and 25 ml. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution added slowly with stirring. This procedure is repeated five times. The euglobulin fraction is dialyzed against saline until free from sulfate and then the water-insoluble euglobulin precipitated by dialysis in the refrigerator against frequent changes of distilled water. After removal of the water-insoluble euglobulin precipitate, merthiolate is added to the supernatant to a final concentration of 1:10,000 and the solution filtered through an L2 Chamberland filter to insure sterility. All precipitations and centrifugations should be carried out at icebox temperatures if possible and a drop of toluene should be present to prevent bacterial and mold growth, especially during dialysis.

Alcohol Fractionation: Cohn and his co-workers (5) have developed methods for the fractionation of the various serum proteins by equilibration through membranes with ethanol-water mixtures under controlled conditions of pH, ionic strength and temperature. Their method for the preparation of gamma globulin from beef plasma is as follows:

Two liters of plasma are diluted with an equal volume of 0.9 per cent sodium chloride solution, and chilled to 0° C. Fibrinogen and fibrin are removed by addition of ethyl alcohol to a final concentration of 10 per cent by volume. The gamma globulin fraction was then separated by increasing the volume fraction of alcohol to 20 per cent, the temperature being maintained at 0° C. Addition of alcohol in each instance is carried out by the McMeekin technic (see III, 33) of equilibration through membranes with alcohol-water mixtures. The alcohol concentration within the membrane never exceeds 50 per cent by volume. When the alcohol concentration in the plasma reaches 20 per cent, the precipitate is removed by centrifugation in a refrigerated centrifuge at -5° and washed with an alcohol-water mixture of the same composition as the solution from which it has been separated. The washed precipitate is then mixed with a further small amount of this ethanol-water mixture, frozen in a thin layer on the sides of the large centrifuge bottle in which it had been separated and lyophilized (see III, 34). The dried pure white readily soluble protein is ground with pestle and mortar and dried further in a vacuum desiccator. The electrophoretic analysis of this material dissolved in phosphate buffer at pH 7.7 of ionic strength 0.2, indi-

cated that the product is very largely γ -globulin, with a trace of faster moving component.

TABLE 1
Properties of Gamma Globulins from Several Animal Species

	Sedimentation Constant S_{20}		Diffusion Constant $D_{20} \times 10^7$	Molecular weight	Frictional ratio f/f_0	Isoelectric point pH
	% protein	Svedbergs				
Human (2,6-8)	0.63	7.1	cm ² /sec 3.84	177,000	1.5	6.6
Bovine (7)	1.0	6.4, 17				
Horse (7)	1.0	6.2, 17				
Rabbit (1,6,8)	0.53	6.5	3.75	165,000	1.6	5.8 ₅

Electrophoretic mobility at pH 7.7 $u \times 10^5$	Proportion of gamma globulin in normal serum†	Minimum amount of N to give		
		a 5 colloidal gold reaction	a +++ cephalin flocculation	anticomplemen- tary effect with 2 units of complement ¹
cm ² /volt-sec -0.9*	% 13	μg 0.2-1	μg 50	μg 34-90
-1.6*	22			
-1.9*	24			
-1.2†	17			

* 1% protein in phosphate buffer, ionic strength 0.2

† 0.02M phosphate buffer +0.15M NaCl

‡ From electrophoretic patterns.

|| Conditions of test in references (2,8).

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CHAPTER 39

CRYSTALLINE OXYHEMOGLOBIN (1)

Crystalline oxyhemoglobin also offers a well-defined species-specific protein suitable for immunochemical studies. Oxalated or defibrinated dog or horse blood of known oxyhemoglobin content is centrifuged and the plasma or serum and the layer of white cells are removed. The red cells are then washed three times with chilled 0.9 per cent sodium chloride solution, after which the supernatant liquid usually gives at most only a faint haze when a test portion is boiled. The cells are then rinsed into a flask with a few ml. of water. The vessel is cooled in ice water, and a steady stream of a mixture of 4 parts of carbon dioxide to 1 part of oxygen passed into the contents. Toluene is, meanwhile, added in amount equal to about one-seventh of the volume of corpuscles, and the mixture is stirred with the gas inlet tube until it becomes pasty. Passage of the gas is continued for a few minutes, with vigorous stirring, after which the flask is stoppered tightly with a rubber stopper and allowed to stand over night in the icebox. This is often long enough to complete the process of disintegration of the cells and crystallization of the oxyhemoglobin, but if many intact cells are still to be seen under the microscope the treatment with carbon dioxide and oxygen is repeated and the flask allowed to stand a day or two longer.

The consistency of the resulting mixture depends somewhat upon the extent to which the red cells have been packed in the centrifuge and upon other factors which have not been determined. If the mixture is sufficiently thin it may be centrifuged in a refrigerated centrifuge, separating into an upper layer of toluene and cell fragments, an intermediate layer of clear solution, and a lower layer of oxyhemoglobin crystals. The two upper layers are poured off and the crystals drained in the icebox on a chilled porous plate, the surface layer being renewed constantly as it dries out, in order to avoid possible conversion of the oxyhemoglobin into a form in which the oxygen is less reactive. During this process a slow stream of carbon dioxide should be directed over the surface of the plate, otherwise a portion of the oxyhemoglobin will redissolve as carbon dioxide is given off from the mixture. When drainage is as complete as possible, the oxyhemoglobin is scraped into a chilled mortar and ground to a smooth

paste with sufficient ice-cold water to bring the final volume up to three to three and a half times (in ml.) the weight in grams of oxyhemoglobin present in the original blood.

In case the crude mixture of crystals, toluene, and cell fragments is too thick to permit centrifugation, the entire mass is transferred to a porous plate, using the same precautions as given above. Under these conditions the process of drainage takes much longer and cannot be carried to completion owing to the emulsion formed by the toluene. On the other hand, the product, being less compact, is easier to grind to a smooth paste with water, and toluene and cell fragments may be removed during the first recrystallization. The final volume in this case should be kept as close as possible to that given above.

The thin paste of crude oxyhemoglobin is transferred to a beaker, set in ice water, and titrated to minimum turbidity with normal sodium carbonate solution. During the addition of carbonate the mixture is stirred thoroughly, and any lumps which may remain are disintegrated. The amount of sodium carbonate necessary is greatest, of course, when the crude crystals have been thoroughly drained and contain as little as possible of the bicarbonate and salts of the mother liquor. In this case the final concentration of alkali added as carbonate is approximately 0.1*N*. If the toluene and cell fragments have been separated previously by centrifugation and if enough water is present, a fairly clear, deep red solution will result, but if too little water is used a crystalline precipitate of what appears to be sodium oxyhemoglobinate will remain. In this case, and also in the case in which the toluene and cell fragments are still present, the carbonate solution is added to the point of minimum turbidity, after which 1 or 2 ml. more are added in order to make sure of an excess.

The solution is next centrifuged, and any toluene and cell fragments on top are sucked off through a capillary tube, a process which can generally be accomplished without appreciable loss of the actual oxyhemoglobin solution. If loss should occur, however, the mixture which has been sucked off may be whirled again and the clear oxyhemoglobin solution added to the main portion. If enough alkali has been added and there is still a crystalline deposit in the centrifuge tubes, too little water is present, and the precipitate may be dissolved in the minimum amount of water and the solution added to

the main portion. This precipitate, which is usually encountered at this point only when dog blood has been used, appears to be sodium oxyhemoglobinate, for it is readily soluble in water with a bright red color, it has a characteristic crystalline form, and finally, yields crystals characteristic of dog oxyhemoglobin when a concentrated aqueous solution is saturated with carbon dioxide-oxygen mixture and allowed to stand in the cold.

The oxyhemoglobin solution is next chilled and a stream of the carbon dioxide-oxygen mixture passed in until crystallization begins, after which the flask is tightly stoppered and set in the ice box. Often within a few minutes the oxyhemoglobin has set to a solid cake of long, flat, scarlet needles in the case of dog oxyhemoglobin, and dark red, glistening, broader plates, often diamond-shaped or hexagonal, in the case of horse oxyhemoglobin.

After standing over night in the icebox the crystals are centrifuged off in the cold or are sucked off on hardened paper in a Buchner funnel (the 5 inch size is adequate for the oxyhemoglobin from 300 ml. of blood). The filtration is carried out in the icebox, with a slow stream of carbon dioxide passing into the funnel. The surface is kept moist by renewal with a spatula as it dries out, and when this is no longer possible, a few ml. of water saturated with carbon dioxide are sucked through with the same precautions, after which the filtration is stopped. The entire filtration usually takes less than 1 hour.

For many purposes the oxyhemoglobin is undoubtedly sufficiently pure at this point, and in one experiment which was interrupted at this stage the amount of crystalline oxyhemoglobin recovered was 46 per cent of the amount present in the original blood, as determined by the oxygen capacity.

For further purification the recrystallization process is repeated. The crystalline cake is transferred to a chilled mortar and again ground to a smooth paste with cold water. The volume of the suspension thus obtained should be about 0.7 of that employed for the first recrystallization if the toluene and cell fragments have been initially removed by centrifugation, and from 0.4 to 0.6 as large if the removal of the upper layer was accomplished during the first recrystallization. The larger fraction will, of course, be necessary when drainage of the original crystallized cell mixture on the porous plate has been most complete. The suspension of oxyhemoglobin

is dissolved with normal sodium carbonate solution, centrifuged, reprecipitated with the carbon dioxide-oxygen mixture, and collected, at every step with the same precautions as in the first recrystallization.

If salt-free oxyhemoglobin is desired, the crystals are ground with the minimum amount of cold water to a paste which will just flow easily, saturated in the cold with the carbon dioxide-oxygen mixture, transferred at once to narrow collodion (2) or cellophane dialysis bags, (III, 33) and dialyzed under pressure in the icebox against water saturated with carbon dioxide-oxygen mixture.

At the end of the dialysis the contents of the bags, which still retain their crystalline structure, are sucked off in the icebox on hardened paper in a Buchner funnel, using, as before, the precaution of keeping the surface layer moist. The use of carbon dioxide at this stage is unnecessary, as the oxyhemoglobin remains sparingly soluble in the absence of alkali and salts.

A simpler method of preparation which gave very nice crystals with horse hemoglobin and which would probably be satisfactory (3) for horse and donkey hemoglobin has been described by Heidelberger and Landsteiner (4):

Defibrinated horse blood is centrifuged, washed five times with saline and dissolved in a volume of water equal to twice the volume of blood used. Ether is added and the mixture placed in the refrigerator for 1-2 days. The ether should be purified by washing it repeatedly with water to remove peroxides which may be present. The washed ether is dried over calcium chloride and then over sticks of potassium or sodium hydroxide. By using purified ether the formation of methemoglobin is avoided. The solution is decanted from any sediment, and an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution is added. Filtration should be started immediately through several fluted filters since crystallization of hemoglobin occurs so rapidly (3, 4) that some hemoglobin is usually retained with the globulin precipitated on the filter. The filtrate is allowed to stand over night in the cold and the supernate decanted from the heavy deposit of oxyhemoglobin crystals. These are filtered on a Buchner funnel, washed with half-saturated $(\text{NH}_4)_2\text{SO}_4$ and recrystallized following the procedure outlined above.

Ferry and Green (5) have also described a method for the preparation of crystalline oxyhemoglobin.

Hemoglobins have been considered to be relatively poor antigens. Boyd (6) has recently produced good antisera suitable for quantitative precipitin analyses using mixtures with adjuvants including suspensions of *Monilia*.

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CHAPTER 40

THYROGLOBULIN

As an example of a protein hormone containing iodine and showing organ specificity, thyroglobulin has been of considerable interest in immunochemistry (cf. II, 9). Heidelberger and Palmer (1) improved the method of preparation by removal of nucleoprotein in the cold at pH 4.8-5.0, before precipitating the thyroglobulin from neutral solution by half saturation with sodium sulfate. Their procedure is as follows:

Thyroids, taken directly from hogs at the slaughter-house, were dropped into ice water, washed several times with ice water, and let stand over night under ice water to remove blood. The glands were then trimmed, kept in the cold except during the manipulations, cut up, and run rapidly through a meat chopper. The weight of pulp in the instance given was 850 gm. The iodine content was 0.03 per cent, or 255 mg. The pulp was pressed out as thoroughly as possible in a hand-press and the extract was immediately chilled. The cake was stirred with 300 ml. of chilled 1 per cent sodium acetate solution (to provide a small amount of electrolyte), ground rapidly in mortars, and again squeezed in the press. The 850 ml. of press-juices, containing 234 mg. of iodine, or 92 per cent of the amount in the pulp, were diluted to 1 liter, stirred thoroughly with about 100 ml. of toluene to assist in removal of fat, let stand overnight, and whirled in a refrigerated centrifuge. As much toluene and cream as possible was sucked off, and the residual solution was poured from the sediment and found to contain 216 mg. of iodine. 50 per cent acetic acid was then cautiously added until the precipitate which formed seemed at its maximum. The pH was 5.0. The solid was centrifuged off (Fraction A). The 850 ml. of supernatant liquid, containing 185 mg. of iodine, were neutralized to litmus, diluted to 2 liters, and the thyroglobulin precipitated with 2 liters of saturated sodium sulfate solution at 35° C. sodium sulfate being used instead of ammonium sulfate in order to avoid the necessity of dialysis before nitrogen could be determined. Precipitation commenced when 1100 ml. of the sulfate solution had been added. The mixture was run through a Sharples supercentrifuge and the precipitate of thyroglobulin collected (Fraction B). 900 gm. of solid an-

hydrous sodium sulfate were added to the supernatant liquid giving rise to a further precipitate which was again collected in the Sharples centrifuge. Since this fraction contained only 1.8 mg. of nitrogen and 0.017 mg. of iodine, it was discarded. The 3.75 liters of supernatant contained only 237 mg. of thyroglobulin as calculated from the 0.038 mg. of iodine in the heat coagulable protein obtained from 100 ml., or less than 1 per cent of the total.

The thyroglobulin (Fraction B) was dissolved in ice-cold water, centrifuged to remove small amounts of insoluble material, diluted to 1.5 liters, and precipitated with 1.5 liters of saturated sodium sulfate solution at 35° C. The mixture was run through the Sharples centrifuge, resulting in a loss of 6.1 mg. of iodine, calculated as 1.02 gm. of thyroglobulin, in the effluent. The precipitate was redissolved in 1.5 liters of water, and an attempt was made to fractionate it by means of sodium sulfate. A slight turbidity developed in the solution when 700 ml. of warm saturated sodium sulfate had been added. The mixture was centrifuged at room temperature, and since only traces of brownish material had deposited, this was discarded and 200 ml. more sodium sulfate solution were added to the supernatant liquid, bringing the concentration of added sulfate to 38 per cent of saturation. At this point only 5.4 per cent of the total yield was precipitated (Fraction B₁). The addition of only 100 ml. more of sodium sulfate solution (42 per cent saturation) brought down 80 per cent of the thyroglobulin, (Fraction B₂), while the addition of 700 ml. more to the supernatant liquid from this yielded an additional 14.6 per cent (Fraction B₃). The final solution contained nitrogen equivalent to 0.48 gm. of thyroglobulin. The three fractions were filtered through small amounts of paper pulp and Fraction B₂ was run through a Berkefeld filter in addition. Fractions B₁, B₂, and B₃ were practically identical in optical rotation and iodine content, and were almost phosphorus-free. Thus no fractionation of the crude fraction B had been effected, except that fraction B₁ contained somewhat more color than the other fractions. The yields of the three fractions calculated on the basis of 15.8 per cent of nitrogen, were 1.2 gm., 17.5 gm., and 3.1 gm., respectively, or a total of 21.8 gm. Since the original press-juice contained 0.234 gm. of iodine, and the thyroglobulin recovered contained 0.6 per cent of iodine, the yield should have been 0.234 divided by 0.006, or 39 gm. The actual yield was therefore 56 per cent of the theoretical on the assumption

that all of the iodine in the press-juice was thyroglobulin iodine. The yield would be improved by recovery of the thyroglobulin in the discarded portions, especially from the supernatant liquids from the first two reprecipitations of fraction A.

TABLE 1

Properties of Thyroglobulins from Various Species (2-4)

	Hog	Human	Bovine	Sheep
Nitrogen %	15.8			
Iodine %	0.58	0.31; 0.63; 0.75	0.21; 0.68	0.34
Optical rotation $[\alpha]_D$	-57 to -61°			
Sed. Const. Svedbergs	19.2	19.2		
Diffusion Const. = $D \times 10^7$ cm ² /sec	2.4; 2.65			
Mol. Wt.	650,000 \pm 20,000			
Isoelectric point pH	4.58			

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CHAPTER 41

CRYSTALLINE ENZYMES AND ENZYME PRECURSORS

Crystalline enzymes have proven of considerable value in the purification of antitoxins (I, 6; IV, 43) and are also useful for immuno-chemical studies. The procedures developed by Northrop (1, 2) for the isolation of crystalline chymotrypsinogen, chymotrypsin, trypsinogen and trypsin from fresh cattle pancreas are given*.

1. Preliminary Purification and Concentration: Remove pancreas from cattle immediately after slaughter and immerse at once in enough ice cold $0.25\text{ }N\text{ H}_2\text{SO}_4$ to cover the glands. Remove fat and connective tissue and mince in a meat chopper within a few hours. Suspend 3 liters of minced pancreas in 6 liters of $0.25\text{ }N\text{ H}_2\text{SO}_4$ at 5° C. and allow suspension to stand at about 5° C. for 18-24 hours. Strain the suspension through gauze, resuspend the residue in an equal volume of cold $0.25\text{ }N\text{ H}_2\text{SO}_4$, and strain through gauze immediately. Reject residue. Dissolve 242 gm. of solid $(\text{NH}_4)_2\text{SO}_4$ in each liter of combined filtrate and washings. Filter through soft fluted paper in cold room. Reject precipitate. Dissolve 205 gm. of solid $(\text{NH}_4)_2\text{SO}_4$ in each liter of filtrate. The heavy precipitate which forms is allowed to settle for 2 days at 5° C. Decant supernatant solution and filter residue with suction through hardened paper on a large funnel. Yield about 100 gm. precipitate. Reject filtrate. Dissolve each 100 gm. precipitate in 300 ml. water, add 200 ml. saturated ammonium sulfate, stir in 5 gm. Standard Super Cel (Celite Corporation) and filter with suction through soft paper. Reject precipitate. Add slowly 205 gm. solid $(\text{NH}_4)_2\text{SO}_4$ to each liter of filtrate and filter with suction through hardened paper. Precipitate "A" about 90 gm. (mixture of crude chymo-trypsinogen, trypsinogen, and inhibitor). Reject filtrate.

2. Crystallization of Chymo-Trypsinogen: Dissolve each 90 gm. of precipitate "A" in 135 ml. water, add 45 ml. saturated $(\text{NH}_4)_2\text{SO}_4$, then adjust to pH 5.0 (with 0.01 per cent methyl red solution on test plate) by addition drop by drop of about 2 ml. $5\text{ }N\text{ NaOH.}$ Allow to stand for 2 days at $20\text{-}25^\circ\text{ C.}$ A heavy crop of chymo-trypsinogen crystals gradually forms. Filter with suction

*Reprinted from Northrop, *Crystalline Enzymes*, by permission of author and of Columbia University Press.

through hardened paper (Filtrate "Tg"). Wash crystalline filter cake with 0.25 saturated $(\text{NH}_4)_2\text{SO}_4$ (1 vol. saturated $(\text{NH}_4)_2\text{SO}_4$ plus 3 volumes H_2O) and finally with saturated $(\text{NH}_4)_2\text{SO}_4$ and store at 5°C . Yield about 25 gm.

3. Recrystallization: The 25 gm. of crystalline filter cake is suspended in 75 ml. of water and 5 N H_2SO_4 added from a burette with stirring until the precipitate is dissolved. 25 ml. of saturated $(\text{NH}_4)_2\text{SO}_4$ is added and an equivalent amount of 5 N NaOH is then added, with stirring, and the solution is inoculated and allowed to stand at 20°C . Crystallization should be practically complete in an hour.

Conversion of Chymo-trypsinogen to Chymotrypsin; Isolation and crystallization of chymo-trypsin: The chymotrypsinogen should be recrystallized eight times. 10 gm. of crystalline chymo-trypsinogen filter cake is suspended in 30 ml. water and dissolved by the addition of a few drops of 5 N H_2SO_4 . 10 ml. $M/2$ phosphate buffer at pH 7.6 are added and a quantity of molar NaOH equivalent to the acid is also added. About 0.5 gm. crystalline trypsin is added and the solution left at about 5°C . for 48 hours. Any active trypsin preparation (of equivalent activity) may be used instead of the crystalline trypsin. After 48 hours the solution is brought to pH 4.0 by the addition of about 5 ml. N H_2SO_4 , 25 gm. solid $(\text{NH}_4)_2\text{SO}_4$ is added, and the precipitate filtered with suction.

Crystallization: The filter cake is dissolved in 0.75 volume $N/100$ H_2SO_4 and filtered if the solution is not clear. The clear filtrate is inoculated and allowed to stand at 20°C . for 24 hours. About 5 gm. of crystalline filter cake should form.

Recrystallization: The crystalline filter cake is dissolved in 1.5 volumes $N/100$ H_2SO_4 ; 1 volume of saturated $(\text{NH}_4)_2\text{SO}_4$ is then added cautiously until crystallization commences. The solution is allowed to stand at room temperature and practically complete crystallization should take place in about one hour.

A further crop of crystals may be obtained by precipitating the mother liquors with saturated $(\text{NH}_4)_2\text{SO}_4$ and treating the precipitate obtained in this way as described under crystallization.

3. Crystallization of Trypsinogen: Adjust filtrate and washings from chymo-trypsinogen crystallization (Filtrate "Tg") to pH 3.0 (with 0.01 per cent methyl orange on test plate) with about 1 ml. 5 N H_2SO_4 per 100 ml. filtrate. Dissolve 30.4 gm. of solid ammonium

sulfate in each 100 ml. of filtrate and filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (40 gm.) in 120 ml. water, add 80 ml. saturated ammonium sulfate and 2 gm. Filter Cel, and filter with suction through soft paper. Wash paper with 0.4 saturated $(\text{NH}_4)_2\text{SO}_4$. Reject precipitate. Add slowly 100 ml. saturated $(\text{NH}_4)_2\text{SO}_4$ to each 100 ml. of combined filtrate and washings. Filter with suction through hardened paper, size 18.5 cm. or larger. Reject filtrate. Wash precipitate on funnel with saturated MgSO_4 in 0.02 N H_2SO_4 to remove excess of $(\text{NH}_4)_2\text{SO}_4$. The washing with saturated MgSO_4 must be done rapidly, otherwise the precipitate is partly dissolved. Saturated MgSO_4 is poured on the precipitate to a height of about 5 mm. and allowed to filter for a few minutes; then the excess of saturated MgSO_4 is decanted, and filtration is continued until complete. Dissolve precipitate (30 gm.) in 30 ml. 0.4 M borate buffer pH 9.0 at 2-5° C. (in an ice water bath), add more borate buffer drop by drop to pH 8.0, measure volume of solution, and add equal volume of saturated MgSO_4 . Mix and allow solution to stand in ice box at about 5° C. (Solution "B"). Short triangular prisms of trypsinogen appear in the course of 2-3 days. If the solution is inoculated with crystals of trypsinogen, crystallization is much more rapid but the crystals are not so well formed. (If crystallization is delayed more than 4-5 days, or if the material has become partly active during the preparation, crystals of trypsin may appear).

Filter the crystals with suction at 5° C. The precipitate (about 10 gm.) is washed on the funnel several times with cold 0.5 saturated MgSO_4 made up in 0.1 M borate buffer pH 8.0 and finally with saturated MgSO_4 made up in 0.1 N H_2SO_4 at room temperature. The crystals are then dried in an electric refrigerator at 5° C. and stored in the cold. The dried material generally contained about 40 per cent of trypsinogen protein and 60 per cent MgSO_4 .

4. Conversion of Trypsinogen into Active Trypsin and Crystallization of Trypsin: Dried trypsinogen containing dry magnesium sulfate is treated as follows: Dissolve 10 gm. in about 50 ml. of water. Add a few drops of 5 N H_2SO_4 if solution is incomplete. Saturate with excess of crystals of MgSO_4 by stirring for 15 minutes at 25° C., filter with suction. Dissolve precipitate (20 gm.) in 20 ml. 0.4 M borate buffer pH 9.0 at 5° C. Add 34 ml. saturated MgSO_4 . Adjust to pH 8.0 with a few drops of saturated KHCO_3 or 5 N

H₂SO₄ if necessary. Inoculate with trypsin crystals. Allow to stand 2-3 days at 5° C., then filter, and recrystallize for trypsin. Yield about 5 gm. filter cake.

5. Recrystallization of Trypsin: Dissolve filter cake (8 gm.) in 6 ml. 0.02 *N* H₂SO₄. Add a few drops of 5 *N* H₂SO₄ if solution is incomplete. Cool to 5° C., add 12 ml. saturated MgSO₄ and 6 ml. 0.4 *M* borate pH 9.0. Adjust to pH 8.0 with saturated KHCO₃ or 5 *N* H₂SO₄ if necessary. Inoculate. Allow to stand for 1 day at 5° C. Yield 3 gm. filter cake.

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CHAPTER 42

BENCE-JONES PROTEINS (cf, I, 6)

This abnormal protein appears in the urine of a high proportion of individuals with multiple myeloma. It is usually detected in urine by its characteristic behavior of precipitating when warmed to 45-58° C. and redissolving completely or partially on boiling. Demonstration of its presence in mixtures of other proteins such as in serum has been attended with considerable difficulty, but has been accomplished by the combined use of salting out, electrophoretic and ultracentrifugal analysis and by immunological methods (1, 2).

Several different Bence-Jones proteins with different electrophoretic mobilities (1), isoelectric points (3) and sedimentation constants (3) have been obtained (table 1). At least two serologically distinct

TABLE 1
Properties of Bence-Jones Proteins

	Svedberg and Sjögren	McFarlane	Andersson and Pedersen†
Sedimentation Constant, Svedbergs	3.55	3.74†	2.79
Molecular weight	35000		36900
Isoelectric point pH	5.2	6.56	5.46
du/dpHo*	5.8×10^{-8}	4.7×10^{-8}	3.5×10^{-8}
pH stability range	3.5-7.5		4.5-11

Data from (3).

* Slope of the mobility pH curve at the isoelectric point.

† Stated to be the same as serum albumin at the same concentration.

‡ A crystalline Bence-Jones protein described in Z. Physiol. Chem., 1936, 243, 173 by Magnus-Levy

Bence-Jones proteins have been demonstrated (4) and both have been identified in the urine of a single patient (5). Numerous workers have obtained crystalline preparations of Bence-Jones protein from urine. Bayne-Jones and Wilson (6) showed that crystalline Bence-Jones protein behaved as a single antigen immunologically distinct from the serum proteins whereas the non-crystalline forms contained traces of the serum proteins. Electrophoretic examination of non-crystalline preparations obtained from urine frequently shows the

presence of small amounts of albumin. For serological studies it is usually necessary to absorb antisera with normal human serum.

Bence-Jones protein may readily be purified from the urine of of myelomatous patients as follows:

Saturated ammonium sulfate solution is added to urine containing Bence-Jones protein to 40 per cent saturation, the slight precipitate which forms is rejected, and the concentration of ammonium sulfate brought to 60 per cent. The precipitated Bence-Jones proteins are centrifuged off, dissolved and reprecipitated once, redissolved in water and dialyzed in the cold until the dialysate no longer reacts with Nessler's solution.

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CHAPTER 43

PURIFICATION OF ANTIBODIES (cf, I, 6)

SPECIFIC METHODS

This group of methods is based upon the removal of the antibody from the serum by specific combination with antigen, followed by dissociation of the antibody from the specific precipitate or agglutinate.

Dissociation of pneumococcal specific precipitates or agglutinates with strong salt solution: The observation by Heidelberger, Kendall, and Teorell (1) that the same amount of pneumococcal polysaccharide precipitated less antibody nitrogen in 1.79 *M* NaCl than in 0.15 *M* NaCl provided the basis for the development of methods for the purification of anticarbohydrates by dissociation of specific precipitates (2) or agglutinates (3) with strong salt solution. With these methods solutions of pneumococcal anticarbohydrate from a number of animal species were obtained in which up to 100 per cent of the total nitrogen was antibody (I, 6). By ultracentrifugal analysis at each step in the process of purification it was shown that the salt dissociation method did not produce any alterations in the size of the antibody molecules (4).

The procedure for the purification of pneumococcal anticarbohydrate from specific precipitates or agglutinates adapted from (2) and (3) is as follows:

The antibody from 100 ml. of type-specific antipneumococcus serum is precipitated at 0° C. by addition of an amount of homologous polysaccharide calculated to bring the system to the beginning of the equivalence zone or preferably to leave a small excess of antibody (see I, 2). After the precipitate flocculates, the mixture is centrifuged in the cold, the supernatant is decanted and the precipitate evenly suspended in chilled saline and washed repeatedly until the amount of heat coagulable or sulfosalicylic acid precipitable protein is at a minimum, 4 to 7 washings usually being sufficient. The washed specific precipitate is then extracted at 37° C. with about 30 ml. of 15 per cent sodium chloride solution for one hour in the presence of a drop of toluene. After centrifugation the precipitate is again extracted with an additional 15 ml. of the 15 per cent

salt solution. The 15 per cent NaCl extracts are dialyzed in the cold under negative pressure against repeated changes of 0.9 per cent saline in the presence of toluene until the desired volume has been reached. The precipitate usually formed during the dialysis is removed from the supernatant which constitutes the purified antibody solution. The degree of purity of the antibody solution is determined by the ratio of the antibody nitrogen content as measured with the quantitative precipitin or agglutinin methods, to the total nitrogen content of the solution. The yield may be calculated from the amount of purified antibody recovered and the amount present in the volume of serum used.

An additional yield of antibody may be recovered from the precipitate remaining after extraction with 15 per cent salt solution by the use of the barium hydroxide method described below.

The use of polysaccharide may be avoided by preparing specific agglutinates using heat-killed or formalin and heat-treated suspensions of type-specific pneumococci (see I, 3 for preparation).¹ A freshly washed pneumococcus suspension containing about 1 to 3 mg. of bacterial N per ml. is added to 100 ml. of antiserum at 0° C. or at 37° C. in 5 to 10 ml. portions with centrifugation after each addition until agglutination of the added cells occurs only slowly, or until the supernatant gives only a slight precipitin test with the appropriate type-specific polysaccharide. The agglutinated bacteria are centrifuged thoroughly, resuspended smoothly in saline at the appropriate temperature and washed until the amount of heat coagulable protein in the wash liquor reaches a minimum. The washed specific agglutinate is extracted at 37° C. with about 30-50 ml. 15 per cent NaCl solution and the procedure continued as outlined above for specific precipitates.

Satisfactory results have been obtained by the salt-dissociation method with horse, cow, pig, rabbit, monkey and human antipneumococcus sera. Types I, II, III, and VIII specific carbohydrates and the pneumococcus "C" substance gave satisfactory results. The yields of antibody obtained by salt dissociation of specific agglutinates from rabbit antisera were small, but with horse sera yields as high as 30 per cent were realized. While antibody solutions generally ranged in purity from 80-100 per cent, with some sera solutions contained but 40-60 per cent antibody N. Antibody solutions of high and low purity were usually homogeneous electrophoretically

(5) and ultracentrifugally (4) so that differences between these products could not be detected by these methods. The reasons for formation of a high grade antibody solution in one case and not in another are unknown although they appear to be a function of the original serum employed.

The salt-dissociation method is probably applicable to many carbohydrate-anticarbohydrate systems. It cannot be used for protein-antiprotein systems. However, Oudin and Grabar were able to obtain small amounts of anti-egg albumin by this method (5a).

Dissociation of pneumococcal specific precipitates or agglutinates by barium hydroxide and barium chloride: This procedure usually yielded an additional portion of antibody of high purity when applied to the specific precipitate remaining after 15 per cent NaCl extraction. The specific precipitate was washed once with water to remove most of the salt, suspended in water and treated in the cold with 0.5 to 1 ml. more saturated barium hydroxide solution than necessary to dissolve it completely. After one hour in the cold 10 per cent barium chloride solution was added to maximum precipitation (1-4 ml.) and the mixture neutralized with acetic acid and centrifuged. The solution usually contained much antibody and was dialyzed against 0.9 per cent saline until free from barium ion. In the case of specific agglutinates, the residual bacteria did not dissolve but an additional 0.5 to 1 ml. of saturated barium hydroxide was added after the solution was blue to thymolphthalein. Antibody solutions prepared by this technic were usually 80 to 100 per cent precipitable and yields reached 30 per cent of the antibody content of the original serum. Ultracentrifugally homogeneous preparations were obtained with rabbit antibody, but antibody solutions prepared in this manner from equine antisera were found to contain ultracentrifugally inhomogeneous components of lower molecular weight than the homogeneous preparations obtained by salt dissociation (4).

Chow and Wu (6) prepared purified antibodies by suspending washed specific precipitates in water and dissolving with *N*/70 sodium hydroxide. The solution is partially neutralized to pH 7.6 with *N*/70 HCl and solid NaCl added to give a concentration of 0.9 per cent. A precipitate forms which is removed leaving the antibody in solution. Liu and Wu (7) have successfully modified this method for purifying antibody from egg albumin anti-egg-albumin

specific precipitates with recoveries of 40-50 per cent and 96 per cent purity. They suspend the specific precipitate in cold water and add an equal volume of 0.2M NaOH, allow to stand at 0° C. for 1-2 hours, neutralize and add NaCl to a concentration of 0.9 per cent and centrifuge off and discard the insoluble precipitate. These preparations of purified antibody have not been characterized electrophoretically or in the ultracentrifuge, and the effect of the sodium hydroxide has not been evaluated. Changes in sedimentation constant at least as great as those produced by barium hydroxide may have occurred.

Dissociation of Wassermann-type antibody-antigen aggregates with strong salt solution and ether: The method of purifying antibody by extraction of washed specific precipitates or agglutinates (2, 3) with 15 per cent NaCl solution was applied by Bier and Trapp (8) to the purification of Wassermann antibody removed from syphilitic human sera by absorption on the usual flocculating antigens (see I, 3). The yields of antibody, however, were very small. Better results were obtained by extracting the 15 per cent salt suspensions of the floccules with ether (9). The procedure was as follows:

Freshly centrifuged un-inactivated serum (4 to 100 ml.) was mixed with $1/8$ to $1/2$ its volume of a saline suspension of Kahn or Mazzini antigen and allowed to stand in the refrigerator for $1/2$ hour or more. With high titer sera further standing did not increase the yield, but with low titer sera flocculation was sometimes more marked and subsequent centrifugation easier if the antigen-antibody mixture remained over night in the refrigerator. The floccules were centrifuged in the cold until firmly packed and washed three times with cold 0.9 per cent saline; the first washing consisted of $1/2$ to 1 times the volume of the original serum; the second and third washings were smaller and were free from protein by the sulfosalicylic acid test. The sediment was uniformly resuspended during each washing. The final sediment, which has been collected in a single conical centrifuge tube, was suspended in 1 to 2 ml. of 15 per cent NaCl solution and vigorously shaken with 10 ml. of cold ether which dissolved most of the solid. (5, 10 or 20 per cent NaCl was as effective as 15 per cent NaCl, but 0.9 per cent NaCl or 0.9 per cent NaHCO_3 yielded much less antibody). Where large amounts of antigen were used, a second washing with ether was employed.

After removal of the ether by suction, the tube was centrifuged in the cold for 15 minutes; the remaining solid formed a firm pellicle or an oily layer on the surface, which was removed by suction. The clear solution was dialyzed overnight in the refrigerator in a cellophane bag against saline-phosphate buffer at pH 7.4. The small precipitate which invariably formed during this dialysis was removed, the supernatant constituting the purified antibody solution.

Inactivation was found to decrease the yield from high titer sera, but with a few sera of low titer better flocculation, centrifugation and a higher yield resulted if the serum was first inactivated. In a few cases small scale experiments were performed with varying proportions of antigen and serum to determine the optimum ratio for large scale experiments; it was found that the yield was relatively independent of the ratio over a wide range. An amount of antigen slightly less than that necessary to remove all the antibody from the serum seemed easier to manipulate. In general $1/4$ to $1/2$ the volume of antigen was used for the high titer ($1/16$ - $1/64$) sera and $1/8$ the volume for the weaker sera. The purity of the antibody solutions was determined by a modification (9) of the micro-quantitative precipitin technic of Heidelberger and MacPherson (see I, 2, 3) using Kahn antigen. The titer of the purified antibody in the usual flocculation tests was found to be increased 2 to 4 fold by addition of serum albumin or inactivated normal serum. The yields from high titer syphilitic sera represented 8-20 per cent of the total antibody. 0.5 to 2.5 micrograms of antibody protein from 15 syphilitic sera in the volume of 0.05 ml. used for the Mazzini test gave a 4+ reaction. Although syphilitic serum usually flocculates poorly if at all unless first inactivated, purified antibody prepared from uninactivated serum gave strong flocculation reactions without inactivation (9).

An alternative method of obtaining purified antibody suspends the washed floccules in absolute alcohol cooled in an ice-salt mixture followed by centrifugation of the floccules in the cold and extraction with ether and drying. The residue is dissolved in saline-phosphate buffer; any insoluble residue is discarded (9).

Purified antitoxins from toxin-antitoxin floccules by enzymatic digestion: Northrop (10) has described in detail a procedure for the purification and crystallization of diphtheria antitoxin. The method involves digestion of toxin-antitoxin floccules

with trypsin at pH 3.5, followed by ammonium sulfate fractionation. The yield of but 0.3 per cent of the total antitoxin in crystalline form necessitated the use of very large initial volumes of toxin and antitoxin. From the lower sedimentation constant and molecular weight it probably represents a degradation product of antitoxin (I, 6).

Both Pope and Healey (11) and Petermann and Pappenheimer (12) were able to prepare antitoxin of high purity by peptic digestion at pH 3 of washed toxin-antitoxin floccules from pepsin-treated pseudoglobulin.

NON-SPECIFIC METHOD OF PURIFYING ANTIBODIES

Specific methods of obtaining pure antibodies have as yet only given satisfactory results with anticarbohydrate and Wassermann antibodies. They have not been found applicable to protein-anti-protein specific precipitates with the exception of diphtheria toxin-antitoxin and anti-egg albumin (7). However, several non-specific procedures are available based on separation of the globulin from the other serum proteins. If only those sera are used in which a high proportion of the total serum protein is antibody, globulin solutions in which the antibody constitutes 40-80 per cent of the total protein may be obtained. Two methods are available for selection of suitable sera. The antibody N and the total N or total globulin N may be determined and the proportion of antibody to total protein or globulin may be calculated. With a Tiselius electrophoresis apparatus, the electrophoretic patterns of 1:4 diluted serum and a similar dilution of serum from which the antibody has been absorbed by precipitation with antigen (fig. 87) may be compared and the ratio of antibody to total gamma globulin may be calculated:

$$\frac{\text{area } \gamma\text{-globulin} - \text{area } \gamma\text{-globulin after absorption}}{\text{area } \gamma\text{-globulin}} = \frac{\text{antibody globulin}}{\text{total } \gamma\text{-globulin}}$$

If the areas under the albumin before and after absorption of the antibody are not the same, as may frequently occur as a result of dilution during dialysis, it is necessary before using the above formula to correct one of the globulin areas by multiplying by the ratio of the areas of two albumin peaks which are unaffected by removal

of the antibody. With this method Tiselius and Kabat (5) calculated that 75 per cent of the gamma globulin of a rabbit anti-egg albumin serum was antibody and on separation of this component electrophoretically, a solution containing 76 per cent antibody was obtained. The technic of separating gamma globulin is described in IV, 38.

Concentration of rabbit antibodies by sodium sulfate precipitation: Heidelberger, Turner and Soo Hoo (13), prepared globulin from types I, II and III antipneumococcal rabbit sera for therapeutic use by precipitation with sodium sulfate:

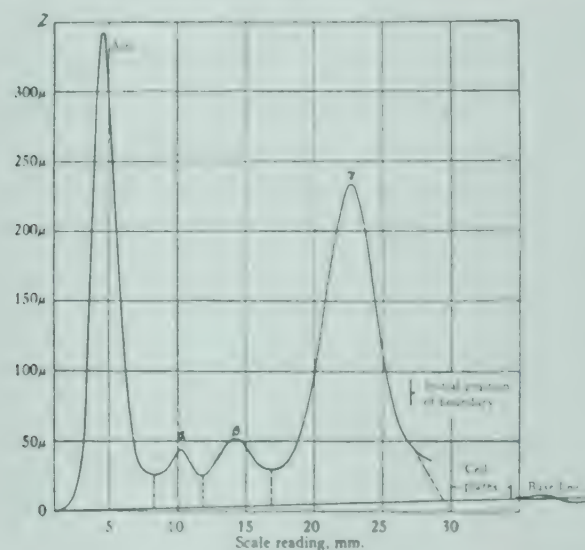


FIG. a

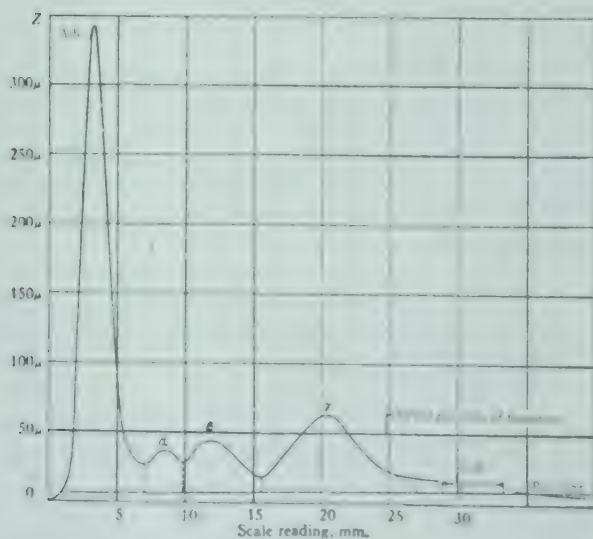


FIG. b

FIG. 87. Electrophoresis scale diagram of anti egg albumin rabbit serum 431:5 1:4 before (a) and after (b) absorption of the antibody. *From (5).*

Sera and solutions were kept sterile, the water and 0.85 per cent saline solution used were prepared with the precautions essential for safe intravenous use. Anhydrous sodium sulfate of the highest purity was sterilized by dry heat before use. The rabbit sera were allowed to stand in the cold for some weeks and freed from separated lipids and sediment. Antisera of a single type, containing 1.0 mg. of type-specific anticarbohydrate nitrogen or more were pooled, diluted when necessary to reduce the antibody N content to 1.0 to 1.5 mg. per ml., and precipitated at 35-38° C. with an equal volume of filtered sodium sulfate solution saturated at the same temperature. After centrifugation at 3000-3500 r.p.m. the clear supernatant was drawn off and discarded; the precipitate was broken up and recentrifuged until no more liquid could be obtained. The tightly packed precipitate was dissolved in water and the solution run through a sterile Chamberland L2 filter. One per cent by volume of a one per cent solution of sodium ethyl mercurithiosalicylate was finally added. The solution may be heated at 56° C. For serological studies but not necessarily for use in patients the sodium sulfate may be removed by dialysis against 0.9 per cent saline. With 6 lots of sera yields of 76 to 89 per cent of the antibody N present in the original serum were obtained and 19 to 40 per cent of the total N of the purified globulin solution was antibody N. The method may be used with all kinds of rabbit antisera, e. g. anti-Hemophilus influenzae Type B (13a).

Concentration of horse antipneumococcal antibody by Felton's method: By this simple method, horse antipneumococcal antibody may be purified so that, in a single step, solutions are obtained in which as much as 65 per cent of the total N is specifically precipitable with homologous type-specific polysaccharide.

100 ml. of horse antipneumococcal serum is poured slowly into 2 liters of cold distilled water with continuous stirring. A white precipitate forms which usually flocculates and settles on standing overnight in the refrigerator, leaving a clear or faintly opalescent supernatant. Occasionally the precipitate does not settle readily and it is necessary to add 2 ml. of *M* phosphate buffer at pH 6.8 per each 2 liters of water used. The precipitate is centrifuged off in the cold and packed as tightly as possible. It is then uniformly suspended in about 15 ml. of cold distilled water and dissolved by addition of 10 ml. of 10 per cent NaCl solution. The solution is finally diluted

to 100 ml. with distilled water, including 1 ml. of a solution of 1 per cent sodium ethyl mercurithiosalicylate as a preservative, and is now isotonic. It should be kept at 37° C. for 2 hours, centrifuged to remove any precipitate which forms (14a), and filtered through a sterile L2 Chamberland filter. The method has also been used successfully with pig antipneumococcal sera (4).

Preparation of antitoxic pseudoglobulin: Horse antitoxins may be concentrated by separation of the pseudoglobulin fraction by precipitation with ammonium sulfate and dialysis as described by Pappenheimer, Lundgren and Williams (15):

900 ml. of antitoxic horse plasma containing 1440 units per ml. were treated with 450 ml. of distilled water and 1350 ml. of saturated ammonium sulfate. After standing overnight in the cold the precipitate (total globulin) was collected, dissolved and dialyzed against running water for 2 days. The euglobulin which precipitated on dialysis was centrifuged and washed once with distilled water and discarded. The supernatant and washing, measuring 550 ml., were treated with 275 ml. of saturated ammonium sulfate (1/3 saturation) and left over night in the cold. The precipitate was centrifuged, collected and dissolved in water. After dialysis against running tap water for 48 hours followed by changes of distilled water and removal of precipitated euglobulin, this fraction contained a total of 233,000 units and 18.9 per cent of the N was specifically precipitable by toxin. The main supernatant after removal of the precipitate formed at one-third saturation was brought to one-half saturation with ammonium sulfate. The precipitate was centrifuged off, dissolved and dialyzed as above. The pseudoglobulin fraction thus obtained contained 800,000 units or 61.5 per cent of the total antitoxin of the original plasma. 32.5 per cent of the nitrogen was specifically precipitable by toxin. Electrophoresis showed that this preparation contained 25 per cent of alpha globulin which was devoid of antitoxic activity. The main component had a mobility of -2.6×10^{-5} cm²/volt-sec. in phosphate buffer of 0.1 ionic strength at pH 7.35 which would place it between the beta and gamma globulins. The molecular weight of the antitoxin after removal of the alpha globulin was found to be 184,000 (I, 6). An alternate method for preparing antitoxic pseudoglobulin is given by Wadsworth (16).

Digestion of horse antitoxic pseudoglobulin by pepsin: Diphtheria antitoxin may be split by peptic digestion (17, 11) to

yield active antitoxic material of smaller molecular weight than the original pseudoglobulin. Petermann and Pappenheimer (12) used antitoxic pseudoglobulin prepared as described above. To a 1 per cent solution of this material containing 400 antitoxin units per ml. in 5 per cent of sodium chloride, solid citric acid was added until the pH was 4.2. Then 1 mg. per ml. of crystalline pepsin prepared according to Philpot (18) was added and the solution allowed to stand at room temperature for 30 minutes. It was then heated at 58° C. for 45 minutes, and filtered or centrifuged to remove the coagulated protein. To check further peptic digestion the solution was then neutralized by the addition of powdered sodium tetraborate or by dialysis overnight in the cold against phosphate-borate buffer at pH 8.6. The digested material showed a greatly decreased flocculation time (cf. 19). It contained 0.34 mg. *N* per 300 flocculating units. Its nitrogen was still only 33 per cent precipitable by toxin. It was found to have a molecular weight of 98,000 (12).

Antibody containing concentrates of human gamma globulin have been prepared by ethanol fractionation at low temperature (20). Two globulin fractions, designated as γ_1 and γ_2 , have been described; the former has a mobility between beta and gamma globulin (10) and the latter is the usual gamma globulin.

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CHAPTER 44

AZO PROTEINS

The coupling of diazo compounds to proteins has been one of the most widely used procedures for studying immunological specificity in relation to chemical structure. In most instances, the diazo compound was coupled to complex mixtures of proteins such as horse serum or chicken serum. However, use of a single purified protein is desirable since information may then be obtained about the effect of the introduced group on the specificity of the original protein in addition to the new specificities produced by the azo grouping, and because it then becomes possible to use quantitative immunochemical methods. For preparations made with mixtures, see Landsteiner (1).

The procedure given below describes the preparation of a highly colored diazo compound which has been successfully coupled to crystalline egg albumin (2) and horse serum albumin (3) to give red azo protein dyes. These azo proteins have been used in studies on the mechanisms of immune reactions, since the antigen content of their washed antigen-antibody precipitates could be estimated colorimetrically (4). They have also been of value in studies on the specificity relationships between the original native proteins and the azo proteins (5, 3).

Preparation of R-salt azobiphenyl diazonium chloride: 0.46 gm. of benzidine was dissolved in 100 ml. of water containing 3 ml. of 6*N* hydrochloric acid and tetrazotized at 7-8° C. with an aqueous solution of 0.35 gm. sodium nitrite and the mixture stirred until no test for free nitrite was obtained with potassium iodide and starch. The solution was poured into a chilled solution of 3 gm. of crystalline sodium acetate in 500 ml. of water and to this was added a solution of 0.87 gm. of R-salt (sodium salt of β -naphthol 3:6 disulfonic acid) in 100 ml. of water. The mixture slowly reddened, but the coupling was not completed until 20 ml. of normal potassium carbonate had been added. The mixture was allowed to stand in ice water for 1 hour before use. The colored solution now contained tetrazotized benzidine coupled on one side to R-salt but with the other diazo group free.

R-salt azobiphenylazo-egg albumin (DEa) (2): Six grams of three times recrystallized egg albumin which had been dialyzed free from salts were made up to one liter and 40 ml. of 2*N* potassium carbonate solution was added. 100 ml. of the above diazo solution were then run in every 10-20 minutes as tests made by adding a few drops of the solution to carbonate containing R-salt showed coupling to be complete. After a total of 600 ml. of diazo solution had been run in, 20 ml. of 2*N* potassium carbonate solution were added. Egg albumin is capable of combining with somewhat more diazo solution than the amount actually used, and 10 per cent more may be used to advantage. It is considered advisable not to continue the coupling process to the limit, as, in other cases, this resulted in insoluble complexes. Potassium carbonate was used since potassium salts of the diazo component and the coupled protein dye appeared to be less easily salted out at the above concentrations than did the sodium salt.

The dye protein solution was next chilled and acidified with acetic acid until flocculation first occurred, the optimum pH range varying from 4.6 - 4.2 in different preparations. The supernatant on acidification with more acetic acid, yielded less highly colored material which was either discarded or added to a subsequent preparation.

The crude R-salt azobiphenylazo egg albumin was collected by centrifugation, dissolved in about 750 ml. of water with the minimum amount of *N* Na₂CO₃ solution, centrifuged to remove a small amount of violet-colored insoluble material, and again acidified with the minimum amount of acetic acid required for flocculation. All operations were conducted in the cold and a refrigerated centrifuge was used. In this way, small amounts of less highly colored protein, reactive with anti-egg albumin serum, generally remained in solution, so that under optimal conditions 20 to 25 repetitions of the process resulted in recovery of the main portion of the azo protein as a clear purplish red solution which no longer reacted with anti-egg albumin serum even when solutions as strong as 0.2 per cent were used, indicating that no unchanged egg albumin was present.

In order to remove non-protein dye impurities, the mixture was centrifuged as sharply as possible after the fifth or sixth precipitation and stirred in a freezing mixture with 10 to 20 times its volume of chilled acetone for 1½ hour. After centrifugation (always in the cold), the dark red supernatant was discarded and the precipi-

tate taken up in cold water, redissolved and reprecipitated as before. The acetone treatment usually left a small portion of the dye insoluble.

As a final step, the dye solution was ultrafiltered in the ice box through a 4 per cent collodion membrane and washed continuously with sterile 0.9 per cent saline (III, 33). A drop or two of toluene was also added to the saline. The washings were colorless at first owing to absorption of the dye by the porous earthenware support of the membrane, but eventually much colored material passed through and this was not precipitable by acetic acid or by antiserum to the dye. Washing was discontinued when the color of the filtrate became less than that of a 1:100,000 solution of the dye. The filter contents were then centrifuged to remove traces of violet precipitate and the dye solution was analyzed for nitrogen by the micro-Kjeldahl method. The azo protein was found to contain 14.6 per cent of nitrogen on the ash-free basis. The solution was kept sterile in the ice box by the occasional addition of a drop of chloroform.

In the above preparations rather drastic methods were used, as a product was desired in which egg albumin specificity had been completely abolished. Possibly this was at the expense of denaturation of the egg albumin portion of the dye (6) (cf. II, 10). In a subsequent preparation in which 10 per cent more dye component was coupled to the egg albumin than used above, the DEa was precipitated with acetic acid, redissolved with a slight excess of K_2CO_3 and fractionated with warm saturated sodium sulfate solution. The fraction precipitable at 33 per cent saturation, and on reprecipitation, at 30 per cent saturation, reacted very little with anti-egg albumin serum and was purified by ultrafiltration and finally dried from the frozen state. The fractions precipitable from the Na_2SO_4 supernatants gave strong cross-reactions with anti-egg albumin sera (7).

R-salt azobiphenylazo horse serum albumin (DSa) (3): To 1 gm. of three times crystallized horse serum albumin (Sa) in 250 ml. of water, 10 ml. of 2*N* K_2CO_3 were added and 150 ml. of the diazo solution were run in dropwise with constant stirring. During the addition it was necessary to add 2*N* K_2CO_3 several times, a total of 35 ml. being used. At this point addition of diazo solution was stopped since a preliminary test on a small portion showed that an insoluble compound was formed at the equivalent of 200 ml. of diazo solution. After coupling was complete, the solution was chilled

and acidified with acetic acid to pH 4.7 to precipitate most of the dye protein. The precipitate was suspended in about 150 ml. of cold water and 5 per cent Na_2CO_3 solution was added until solution was as nearly complete as possible. The solution was centrifuged in the cold and the residue discarded. Precipitation with acetic acid left a colorless supernatant which on neutralization, gave no reaction with anti-Sa. The fractions precipitated by acetic acid, however, reacted strongly with anti-Sa. In an effort to fractionate so as to obtain a portion which did not react with anti-Sa, the precipitate was dissolved as before and 130 ml. of water and K_2CO_3 and 65 ml. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution were added. The resulting precipitate was centrifuged in the cold (fraction 1). To the supernatant 33 ml. additional saturated $(\text{NH}_4)_2\text{SO}_4$ solution were added and the precipitate (fraction 2) was centrifuged off. The supernatant was acidified with acetic acid yielding a precipitate, fraction 3. All fractions still reacted strongly with anti-Sa. Fraction 1 was redissolved and given two reprecipitations at $1/3$ saturation with $(\text{NH}_4)_2\text{SO}_4$, after which the supernatant was quite light in color. The final precipitate was centrifuged off sharply, and suspended in 20 volumes of cold redistilled acetone. The precipitate was centrifuged and washed with cold acetone until the washings remained colorless. The residue was suspended in water, dissolved by addition of K_2CO_3 solution, neutralized and filtered through a Chamberland L2 filter. The material was ultrafiltered as described above for the egg albumin azo-dye. The ultra-filtered material still reacted with anti-Sa, but failed to precipitate at any dilution with an antiserum to R-salt azobiphenylazo egg albumin. All quantitative studies were carried out with fraction 1 (3) (see II, 10); fractions 2 and 3 were not studied.

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CHAPTER 45

ACETYLATED AND MALONYL PROTEINS

Acetylated Proteins: Ketene, $\text{CH}_2 = \text{C} = \text{O}$, has been widely used in acetylation of proteins, especially those with biological activity such as enzymes, antibodies, and viruses, and has provided interesting data on the relationship of the free amino and tyrosine hydroxyl groups to biological activity (1, 2, 3, 4).

Ketene reacts with primary and secondary amines, aliphatic alcohols, water, and phenols to form acetyl derivatives. The addition of ketene to a primary amine may be represented as follows:



Primary amines react most rapidly and phenols least rapidly with ketene. Addition of ketene may take place in acid solution, but proceeds more rapidly at alkaline reactions.

Herriott (5) has described a convenient generator for preparing ketene by pyrolysis of acetone vapor (fig. 88) according to the equation



It is constructed of Pyrex glass and has a removable filament support so that a burned out filament may be replaced in a few moments. Platinum wire (Brown and Sharp #30 gauge) across a 110 volt circuit is used with a resistance in series regulated to let 2 to 6 amperes through the filament so as to vary the rate of ketene production. The generator is used with a 500 ml. flask half filled with acetone and containing a few marble chips. The apparatus is placed on a steam bath. From the generator outlet, the acetone and ketene pass into a reflux condenser where the acetone vapor is condensed and returned to the generator and the ketene is led into the solution to be acetylated. Before starting the current, it is essential that the acetone boil for several minutes so that all the air is expelled from the generator. Otherwise the filament may burn out.

Acetylation is usually carried out by passing a stream of ketene into the protein solution to be acetylated, kept at room temperature or 0°C . The protein may be dissolved in an appropriate buffer. For work near neutrality sodium acetate solution or a phosphate buffer may be used. As the reaction proceeds, the solution will

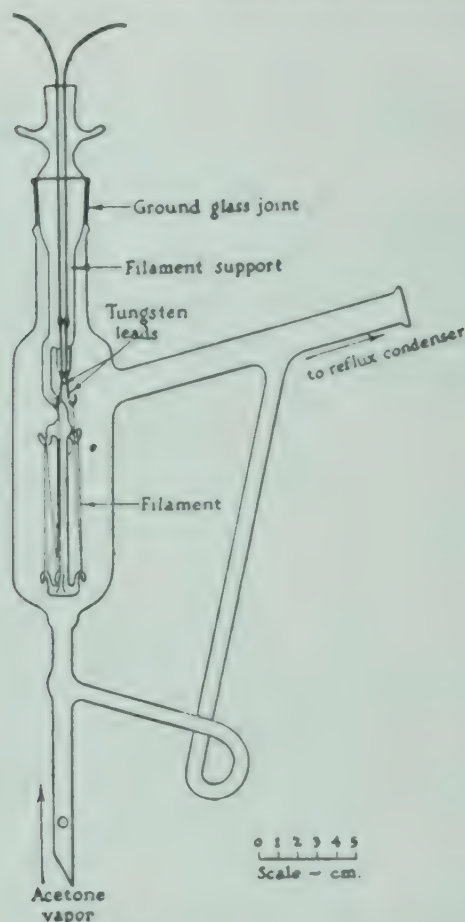


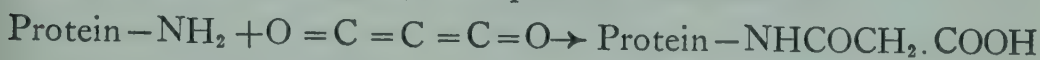
FIG. 88. Ketene Generator. From (5).

become more acid and the rate of acetylation will decrease. To slow down the pH change, the reaction may be carried out in a dialysis sac, placed in a large volume of buffer solution. Alternately the acetylation may be interrupted, the protein precipitated and redissolved in buffer and acetylation resumed. The solution should be stirred while the ketene is being introduced. Samples may be withdrawn at varying time intervals for estimation of amino N (III, 14), acetyl (III, 15) and biological activity. After the reaction has proceeded for the desired time, the solution may be dialyzed to remove the acetate ion. It may be necessary with any given protein to carry out preliminary acetylations to determine optimum conditions of time, temperature and pH. In some instances insoluble compounds may be formed on continued acetylation.

Herriott has introduced a method for estimating the number of acetylated tyrosine groups by difference between determinations with the Folin-Ciocalteu tyrosine reagent (III, 22) carried out at

pH 8.0 and after liberation of acetyl at pH 11.0. At pH 11, the acetyl groups are split off so that all of the tyrosine reacts with the reagents, while at pH 8.0 only tyrosine molecules containing free hydroxyl groups give a color with the Folin reagent. The difference is taken to be acetylated tyrosine. Any color due to tryptophane does not affect this difference since it will be the same at both pH 8 and 11. A description of the technic appears in (2, cf. 6).

Malonyl Proteins: Carbon suboxide, C_3O_2 . $O = C = C = C = O$, has been used for the introduction of malonyl groups into proteins. It reacts with the free amino and tyrosine hydroxyl groups and is of interest because it substitutes acidic groups in place of basic groups on the protein (cf. also phosphorylated proteins, IV-46). Its reaction with the amino group may be represented as follows:



No immunological studies on malonyl proteins have been carried out as yet, although malonyl derivatives of egg and serum albumin and of pepsin have been prepared.

The procedure is similar to acetylation with ketene: carbon suboxide is prepared as described in (7) and liquified by cooling in solid carbon dioxide.

The gas is introduced into the protein solution through a sintered glass filter by warming a few ml. of the liquid carbon suboxide. 0.3 *N* NaOH is added in small portions during the course of the reaction to maintain the desired pH. Two to six hours are usually required for the completion of the reaction and the products may be isolated by acidification and salting out (8). Details may be found in references (7-14).

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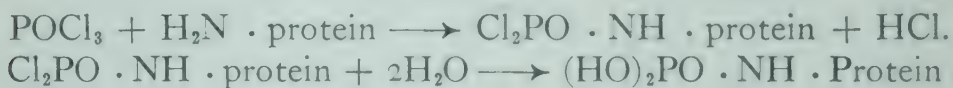
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CHAPTER 46

PHOSPHORYLATED PROTEINS

Immunochemical studies have recently been carried out on the effect of phosphorylation on crystalline hen's egg albumin (1) (PEa) and on crystalline horse serum albumin (2) (PSa). The phosphoric acid groups were introduced with POCl_3 by means of the Schotten-Baumann reaction.

Part of the phosphorus (P) in PSa was shown by Van Slyke amino nitrogen determinations (3) (III, 14) to be linked to free amino groups in the protein, possibly the ϵ -amino groups of lysine. The reaction of POCl_3 with protein probably follows these equations:



These phosphorylated proteins have been found to lose phosphate very readily especially at acid pH and even at refrigerator temperatures. The lability of these phosphoryl groups at acid pH also suggests that they are in amide linkage and not bound to oxygen (i.e. serine, threonine or tyrosine). No direct chemical evidence was obtained, however, which would exclude ester linkage.

PHOSPHORYLATED SERUM ALBUMIN

Method of preparation (2): 1.5 gm. of crystalline horse serum albumin (Sa) (see IV, 37) dissolved in 55 ml. of water and 55 ml. of 6% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ solution are placed in a 500 ml. three-necked flask and chilled in an ice-salt water mixture at 0° to -2°C . 3 gm. of freshly distilled POCl_3 dissolved in 25 ml. of CCl_4 are added dropwise over a period of six hours to the mixture while it is stirred mechanically. Simultaneously, 3 *N* NaOH solution is added drop by drop to neutralize the hydrochloric acid formed and to keep the reaction mixture slightly alkaline to phenolphthalein at all times, corresponding to a pH of 8.5 to 9. Since the use of strong alkali can cause denaturation of the protein, solid sodium or potassium borate may be substituted, but under these conditions less P will be introduced. After addition of the reagents, the reaction mixture is placed

in the refrigerator until the following day. It is then centrifuged in the cold to separate the semi-solid CCl_4 emulsion from the aqueous phase containing the PSa.

The solubility of products prepared using NaOH is sufficiently low at acid pH values to permit precipitation by addition of *N* HCl to about pH 2 to 4. After centrifuging, the precipitate is dispersed in cold water or in 0.9% NaCl solution and dissolved completely by addition of *N* NaOH to pH 5 to 6. Four or five isoelectric precipitations followed by dialysis in the cold against frequent changes of 4-5 volumes of isotonic saline serve to remove most inorganic phosphate. It is frequently preferable to omit precipitation, especially with less heavily phosphorylated and therefore not completely insoluble lots obtained when potassium or sodium borate was used. Free phosphate cannot be removed entirely by dialysis but is only reduced to a constant minimum level. A trace of phosphate (about 0.1 microgram P per ml.) continues to appear in the dialyzate indefinitely, indicating slow spontaneous splitting from the phosphoprotein. The decline in P content with age can be kept to a minimum by storage at pH 8 to 9 in the refrigerator, or, preferably, frozen in solid carbon dioxide. PEa was prepared by essentially the same procedure (1). Some of the properties of the PEa and PSa are given in tables 1 and 2, respectively.

TABLE 1
Properties of Phosphorylated Egg Albumin

Preparation No.	N:P weight ratio of product	N pptd. by 0.09 mg. antigen N from anti-Ea rabbit serum*
5A†	28	mg. 0.22
6A†	12.5	0.08
8A†	9.4	0.10

* N precipitable by 0.09 mg. EaN=0.83 mg.

† Prepared with sodium hydroxide as neutralizing agent.

Unlike the native proteins, phosphorylated egg albumin (PEa) and serum albumin (PSa) were more or less insoluble between pH 2 to 4, the solubility in the case of PSa depending on the phosphorus content. (2). The introduction of the acidic phosphoryl groups caused PSa to migrate more rapidly in an electric field on the alkaline side of the isoelectric point. Chemical combination of the phosphoric

TABLE 2

Properties of Phosphorylated Serum Albumin

Preparation Number	N:P weight ratio of product	Relative fluidity at 1% protein concentration ††	N pptd. by 0.1 mg. antigen N from 1.0 ml. anti-Sa rabbit serum*	Ratio of Amino N to total N†
			mg.	
7 ‡	20.6		0.73	
10P**	13.3	0.920	0.65	
4B††	9.0	0.896	0.46	
5B††	7.2	0.860	0.22	
13 ††	5.9		0.14	0.043

* N precipitated by 0.10 mg. Sa N = 0.86 mg.

† Ratio of amino N to total N in Sa = 0.078.

‡ Prepared with sodium borate as neutralizing agent.

** Prepared with potassium borate as neutralizing agent.

†† Prepared with sodium hydroxide as neutralizing agent.

‡‡ Relative fluidity of 1% Sa 0.962.

acid groups with the protein was indicated by the fact that the phosphorylated protein migrated as a single component (2) with a higher mobility than the native proteins.

The immunological specificity of PEa differed radically from that of egg albumin (Ea) (1). The almost complete failure of Ea to precipitate anti-PEa sera and the similar lack of reactivity of PEa with anti-Ea sera indicated that phosphorylation induced a profound change in molecular structure, as was also noted in the denaturation of Ea by acid (4). Antisera to PEa, however, showed marked cross-reactivity with denatured Ea (DnEa) suggesting that the structural changes caused by phosphorylation resemble those in acid-denatured Ea. The precipitation of DnEa by anti-PEa appeared, however, to be a cross-reaction, and was not due to the presence of DnEa in PEa, since the quantitative relationships were very different from those in the PEa-anti PEa and DnEa-anti-DnEa systems (II, 10).

In the case of PSa, changes in immunological specificity were less extensive (2). PSa cross-reacted with antisera to Sa, the extent of cross precipitation varying inversely with the P content. There was little or no cross-reaction between PSa and antisera to PEa (1, 2). The changes in specificity produced by phosphorylation appeared due to general structural alterations in the protein molecule. In the

case of Ea these were apparently much more pronounced than with Sa, paralleling in this respect the response to these proteins when coupled with an azo dye (cf. IV, 44).

There were immunological indications as well as evidence based on viscosity studies showing that phosphorylation produced some irreversible changes despite the mild procedure employed. Both PEa and PSa solutions were more viscous than those of Ea and Sa, respectively, the magnitude of the viscosity increase depending on the phosphorus content and to some extent on the pH (cf casein) and salt concentration. Such an increase of viscosity may be interpreted as indicating an increase in molecular assymetry as a result of phosphorylation (cf. III, 28). Removal of some of the phosphoryl groups from PSa by mild hydrolysis led to increased reactivity, with anti-Sa and a decrease in viscosity, but the magnitude of these changes was not commensurate with the amount of P removed.

Comparative immunological, chemical and physical studies of a conjugated protein thus show that the changes produced by chemical substitution of a protein depend on the nature of the protein and are not necessarily restricted to the substituent group introduced. There may also be general structural changes in the protein, despite the use of a mild method of conjugation.

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CHAPTER 47

PHENYLUREIDO PROTEINS

The reaction of phenylisocyanate with proteins has been widely used to introduce known chemical structures into proteins so that their effect on immunological specificity could be studied (1-6) in much the same way as had been done extensively with azoproteins (7). Unfortunately, the immunochemical studies have all been conducted with mixtures of proteins such as horse serum globulin so that information could be secured only about the effect on specificity of the group introduced but quantitative immunochemical data could not be obtained concerning the role of the protein molecule itself in specificity. Thus it has been shown that crystalline egg albumin and crystalline horse serum albumin respond in widely different fashion (cf II, 10) to the introduction of azo (8-11) or phosphoryl (12,13) groups (IV, 44, 46).

The reaction between phenylisocyanate and proteins appears to be mainly with the free amino groups of the protein molecule yielding a substituted urea as follows:



The method used by Hopkins and Wormall (1) for the preparation of phenylureido derivatives of caseinogen, gelatin and horse serum globulin avoids the use of drastic conditions which can produce changes other than the introduction of the new groups. The procedure employed for caseinogen is as follows:

To 50 ml. of caseinogen solution at pH 9, containing about 3 gm. of protein, were added 100 ml. of a phosphate buffer of pH 8.0 (250 ml. of 0.2 *M* KH_2PO_4 plus 234 ml. of 0.2 *N* NaOH per 1 liter.) The mixture was then cooled in ice and 2 ml. of phenyl isocyanate added slowly with continuous stirring. The reaction of the fluid was determined at frequent intervals by means of indicators, and 0.2 *N* NaOH was added when necessary to restore the pH to 8.0 approximately. After having been stirred for about 1 hour, the mixture was again adjusted to pH 8.0 and centrifuged, the precipitate (solid diphenylurea) being discarded after one or two washings. 2% acetic acid was then added to the solution and washings to give maximum

formation of a flocculent precipitate. This was removed by centrifuging and redissolved in about 200 ml. of distilled water with the addition of sufficient 0.2 *N* NaOH to give a clear solution. When this and similar precipitates were dissolved in water with the aid of dilute NaOH, the alkali was added very cautiously and frequent tests were made to ensure that the solution did not become more alkaline than about pH 8.5.

Phenylureido-gelatin and phenylureido-horse serum globulin were prepared in the same way except that in the latter case solutions of the globulin were kept isotonic with NaCl. By a similar method p-bromophenylureido compounds of caseinogen, gelatin and horse serum globulin were made by the action of p-bromophenyl isocyanate in ether on a solution of the protein in a phosphate buffer at pH 8.

The products were readily soluble if NaOH solution was added to about pH 7.5 and they were precipitated from solution at about pH 4 to 4.5. The free amino-N content of the derivatives was much lower than that of the native proteins indicating that reaction occurred with the free amino groups of the protein. In the case of p-bromophenylureido derivatives a fairly good correlation was obtained between the bromine content of the derivatives and the decrease in free amino N, indicating that other groups in the protein were probably not involved in the reaction (1).

Qualitative precipitin tests showed that treatment with phenylisocyanate led to a marked change in specificity. Thus antibodies to phenylureido-horse serum globulin reacted very well with the antigen used for injection and with any similarly altered protein (Phenylureido derivatives of chicken serum, rabbit serum, caseinogen and gelatin) and only to a moderate or very slight extent with horse serum globulin. The species specificity had, therefore, been augmented by a specificity characteristic for the phenylureido group. Phenylureido compounds of glycine, alanine and lysine (3) gave marked inhibition of specific precipitation, phenylureido-lysine being especially effective. The latter observation suggested that the reaction with the protein probably involved the -NH_2 group of lysine.

It is of special interest to note that phenylureido-gelatin precipitates with antisera to phenylureido-horse serum globulin, but the former compound, like gelatin, is not antigenic when injected into rabbits (2).

The biological properties of phenylureido derivatives of crystalline tobacco mosaic virus (14) have been studied. These preparations were as homogenous in the ultracentrifuge and the electrophoresis apparatus as the original protein.

Fluorescein isocyanate has been conjugated through the ureide linkage to type 3 pneumococcal antibody (15). The solution of conjugated antibody agglutinated type 3 pneumococci to the same titer as the original serum and rendered the organisms fluorescent in ultraviolet light.

Quantitative immunochemical studies on phenylureido derivatives of purified single proteins would be desirable, to obtain more precise data on the role of the amino group in specificity (cf II, 10).

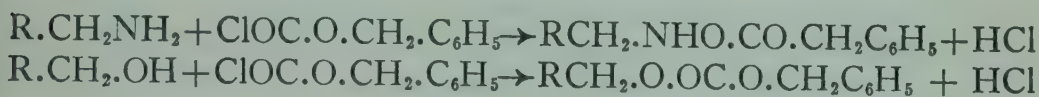
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CARBOBENZOXY AND OTHER ACID CHLORIDE
DERIVATIVES OF PROTEINS

Reaction with carbobenzoxy chloride has also been used to introduce known chemical substituents into the protein molecule at low temperature and at pH close to neutrality. As in the case of phenylureido proteins, interest was focussed on the immunological specificity of the substituent group. Although purified single proteins have been used in some instances, quantitative immunochemical methods have not yet been applied.

Carbobenzoxy chloride (1) reacts with amino- or hydroxy-compounds in cold alkaline solution as follows:



According to Gaunt and Wormall the reaction with proteins (2) appears to take place mainly, if not entirely, between the reagent and the free amino-groups of the protein, as indicated by the disappearance of free amino-N, although no chemical evidence has been given which excludes the participation of the aliphatic and aromatic hydroxy-groups. Bergmann and Zervas' method (1) was modified slightly in (2), the solution being maintained at pH 7.5-8.5 during the reaction to minimize the probability of other changes in the protein molecule.

Preparation of carbobenzoxy chloride: (1) (All operations should be carried out in a good hood since phosgene is highly toxic.) 45 ml. pure benzyl alcohol is added to 240 g. of an ice-cold 20% solution of phosgene in toluol. After $\frac{1}{2}$ hour in ice and an additional 2 hrs. at room temperature, excess phosgene is removed with a stream of dry N₂ or CO₂ gas. Toluol is distilled off *in vacuo*, at a bath temperature of less than 60°C. About 60 g. of acid chloride remains which is sufficiently pure for synthetic purposes.

Preparation of carbobenzoxy-egg albumin: 200 ml. of a 1.7% solution of crystalline egg albumin (3) (IV, 36) were mixed with 200 ml. of phosphate buffer at pH 8, the mixture chilled in ice-water

and 7.5 g. of carbobenzoxy chloride in 30 ml. of ether added slowly with mixing. The mixture was stirred at 5°C for 3 hrs. and then centrifuged. The supernatant was acidified with dilute acetic acid to maximum precipitation of the carbobenzoxy-egg albumin, which was then separated by centrifugation and redissolved in water by adding 2*N* NaOH to pH 8. The solution was centrifuged and the carbobenzoxy protein in the supernatant solution was precipitated by dilute HCl. After two additional precipitations, the carbobenzoxy-egg albumin was dissolved in 0.9% NaCl at pH 7.5. A small amount of chloroform was added as a preservative.

Carbobenzoxy derivatives of gelatin and horse serum globulin were prepared in a similar manner. The carbobenzoxy globulin was completely insoluble and a suspension was used for immunization of animals.

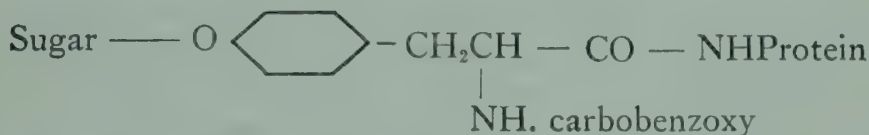
Treatment with carbobenzoxychloride converted egg albumin and gelatin into acid-precipitable derivatives which had a new antigenic specificity. Antisera to the globulin derivatives reacted well in the precipitin test with carbobenzoxy-egg albumin and carbobenzoxy-gelatin, but gave only weak or negative precipitin reactions with unchanged horse serum globulin, indicating almost complete loss of the original species specificity (2). Carbobenzoxyglycine was shown to inhibit the precipitin reaction between carbobenzoxy-gelatin and antisera to carbobenzoxy-horse serum globulin.

Carbobenzoxy derivatives of insulin (2) were prepared by the same procedure except for the use of NaHCO₃ solution instead of phosphate buffer. Carbobenzoxy-insulin was devoid of hypoglycemic activity. Immunization of mice with carbobenzoxy-insulin did not lead to protection against the hypoglycemic effect of insulin. Complement-fixing or precipitating antibodies reactive with carbobenzoxy-insulin or carbobenzoxy-gelatin could not be demonstrated in the sera of these animals (2).

Carbobenzoxy-tobacco mosaic virus (4) was studied by Miller and Stanley who found that in addition to amino groups phenol and indole groups were substituted, although much less extensively. *p*-Chlorobenzoyl and benzenesulfonyl derivatives (4) were also made by similar mild methods. No immunological experiments were reported.

Clutton et al (5, 6) prepared carbohydrate-protein compounds in which the substituent group was bound to the protein in peptide

linkage. The sugar, acetobromoglucose, was first combined with the ethyl ester of N-carbobenzoxytyrosine to form a glucoside on the tyrosine hydroxyl. The acetyl groups were removed in the formation of the hydrazide and the deacetylated compound was converted to the azide and linked to the free amino groups of the protein through the carboxyl group of the tyrosine as follows:



In the case of gelatin the carbobenzoxy group was removed by reduction with sodium in liquid ammonia. The derivative contained 4.6% glucose. The carbobenzoxy residues were removed from the horse serum globulin derivative by catalytic reduction. Similar derivatives of horse serum albumin and insulin were also made but the carbobenzoxy groups were not removed in these instances. The glucosidotyrosyl derivatives yielded rabbit antisera which reacted strongly with the homologous derivative and with the other glucosido-tyrosyl proteins, but failed to give significant precipitin reactions with the original protein.

An alternative method of coupling carbohydrates to proteins through azo linkage has been used by Goebel and Avery (7). The p-nitro-benzyl ether of the sugar is prepared by treatment with p-nitrobenzyl bromide and the nitro group reduced to an amino group which is diazotized and coupled to protein in the usual manner.

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CHAPTER 49

DIPHTHERIA TOXIN

The preparation of diphtheria toxin in a relatively pure and undamaged form has been accomplished (1, 2) by the combined use of several mild chemical procedures for separating the toxin from constituents of the culture medium and from non-toxic components of the bacilli. The earlier methods which were critically reviewed by Eaton (1), especially those involving precipitation with acids or organic solvents, often caused a loss of toxicity and antigenic power, and resulted in products showing an increased flocculation time. Procedures utilizing adsorption, ultrafiltration, salting-out with ammonium sulfate and precipitation of the toxin by metallic salts, have proven less harmful provided the pH of the toxin solutions was maintained between 6 and 9, but combined and repeated use of several of these methods is necessary for effective purification. By adsorption of impurities with calcium phosphate, fractionation of toxin by precipitation with ammonium sulfate, alum and cadmium chloride, and subsequent dialysis, Eaton (1), isolated small quantities of highly purified toxin from a crude toxin produced on peptone medium. His best preparations contained 0.0005 mg. N per Lf* unit and 0.00002 mg. N per M.L.D.**. The availability of a method for producing large quantities of diphtheria toxin on culture media containing only low molecular weight substances enabled Pappenheimer (2) to isolate highly purified diphtheria toxin by a simpler procedure involving only neutral salt fractionation, adsorption of impurities on aluminum hydroxide cream and dialysis. The purest preparation contained 0.00046 mg. N per Lf unit identical within the limits of experimental error with the value found by an independent method based on a quantitative immunochemical study of the flocculation reaction (3).

PROCEDURE (2)

Toxin was prepared in 15 to 20 liter lots on the gelatin hydrolysate medium (4) described below. This medium contained approxi-

*The activity of a toxic solution is expressed in Lf units per ml. An Lf unit is the volume in ml. of toxin solution which gives most rapid flocculation with one "standard" unit of antitoxin (obtainable from the National Institute of Health).

**The minimal lethal dose (MLD) is defined as the amount of toxin solution which, when injected subcutaneously, causes death in four days in 75-80 per cent of a group of guinea pigs weighing 250 gms. each.

mately 1 per cent of a complete hydrochloric acid hydrolysate of Eastman's purified gelatin supplemented by small amounts of methionine, cystine, tryptophane, glucose, maltose, lactic acid, the necessary salts*** and alcohol-soluble accessory factors from purified liver extract. The medium contained 1.8 mg. N per ml. and gave no precipitation with ammonium sulfate at any concentration.

Preparation of Gelatin Hydrolysate Medium: as described in (4)

dl-methionine.....	4	gm.
dl-cystine.....	4	gm.
l-tryptophane.....	1.5	gm.
K ₂ HPO ₄	40	gm.
NaCl.....	80	gm.
MgSO ₄	6	gm.
CaCl ₂ (33% solution).....	6	ml.
CuSO ₄ ·5H ₂ O.....	0.1	gm.
Liver or yeast extract preparation.....	100	ml.
Gelatin hydrolysate.....	500	ml.
Distilled water to.....	20	liters
Sugar + lactate solution (added separately before inoculation).....	400	ml.

The methionine, dipotassium phosphate and sodium chloride are placed in a 6 liter Pyrex Florence flask. 500 ml. of stock acid hydrolysate of gelatin (note 1), 100 ml. of purified liver or yeast extract preparation (note 2) and about 2 liters of distilled water are then added. The cystine is dissolved separately in the least amount of 6*N* hydrochloric acid (note 3) and added to the main lot, which is now made up to 10 liters with distilled water, distributed in three 6 liter Pyrex flasks and brought to pH 7.8-8.0 with 5*N* NaOH. 2 ml. of 33% calcium chloride solution is then added to each flask and the medium boiled gently for 10 minutes (note 4). After it has been allowed to cool for a short time, it is filtered into a 5 gallon Pyrex carboy, the tryptophane, magnesium sulphate and copper sulphate are added (note 5) and it is made up to 20 liters. Finally the reaction is readjusted, if necessary, to pH 7.6-7.8, and the slightly yellow

***It has been shown (6) that the yield of diphtheria toxin in a medium supporting growth of the diphtheria bacillus is highly dependent on the concentration of inorganic iron. While the presence of minute traces of iron stimulates toxin production, amounts in excess of about 0.4 mg. per liter are completely inhibitory, although without effect on growth.

medium distributed into Fernbach flasks (note 6), 600 ml. to each flask, and autoclaved 15 minutes at 120°C. Before inoculation, 12 ml. of a sterile solution containing 37 ml. Merck's c.p. sodium lactate, 15 per cent Merck's purified maltose, 7.5 per cent Merck's c.p. dextrose (note 7) and 0.3 per cent calcium chloride (note 8), are added to each flask.

Notes:

1. The stock gelatin hydrolysate is prepared as follows: 1000 gms. of Eastman's "de-ashed" gelatin is treated with 5 liters of 1:1 hydrochloric acid and autoclaved for 3 hours at 123° C. The deeply colored hydrolysate is evaporated to a thick syrup *in vacuo*, and, after distilled water has been added, again evaporated under reduced pressure. The black syrup is dissolved in about 2 liters of water and adsorbed with 40-50 gms. of decolorizing charcoal. The filtrate is made up to 2500 ml. for stock and is sufficient for 100 liters of medium.

2. The purified liver preparation is made according to Mueller (5). 1 ml. is equivalent to 10 gms. of liver.

The purified yeast fraction may be prepared as follows: 1000 gms. of marmite (vegex) is dissolved in 2.5 liters of distilled water, and a saturated solution of basic lead acetate added until there is no further precipitation. The precipitate is removed and the filtrate is acidified and saturated with hydrogen sulfide. After the lead sulphide has been removed, the filtrate is concentrated *in vacuo* to a small volume (200-300 ml.) and 5 volumes of alcohol are added. The insoluble material is filtered off, the filtrate concentrated to a syrup *in vacuo* and the water-soluble residue made up to 1 liter with distilled water. 5-10 ml. of this solution per liter of medium are sufficient for maximum growth and toxin production. The yeast fraction may be further purified without altering the yield of toxin.

3. Cystine is dissolved separately with HCl, since it otherwise goes into solution only with great difficulty.

4. The purpose of the calcium phosphate precipitation at pH 7.8 is to remove excess iron by adsorption. If all the iron is removed at this point the amount optimal for toxin production may be added later. (See note 7.)

5. Since tryptophane is rapidly destroyed in acid solution, it is not added until after the solution has been neutralized. Magnesium sulfate is not added until this stage in order that none of it may be carried down with the precipitated calcium phosphate.

6. Fernbach flasks made of soft glass (6) have proved more satisfactory than those of Pyrex with this medium, particularly if the medium is allowed to incubate for several days before inoculation. However, 25-30 lf per ml. of toxin may be produced in Pyrex vessels if soft glass flasks are not available.

7. The iron necessary for toxin production (see note 4) is added as impurity in the sodium lactate + sugar solution. The grade of reagents described fortunately contains close to the optimum amount of iron. Should any trouble arise on this score, however, it may be necessary to remove iron from the sugars by the calcium phosphate method at pH 7.8, or from the sodium lactate by the use of redistilled lactic acid, and then determine the quantity of any iron salt which it is necessary to add in order to obtain the highest yield of toxin. The reagents may be tested for their iron content with dipyrindine or thiocyanate. The optimum iron content of the final medium should be approximately 0.05 mg. per liter.

8. Since almost all the calcium previously added has been precipitated as phosphate, a further quantity is added with the lactate sugar solution.

9. The strain of organism is of great importance. Park No. 8 strains vary widely both in toxin-producing capacity and growth requirements, and it is advisable to test a number of Park No. 8 strains to determine the best available.

10. Toxin containing 30-40 lf may be obtained regularly in soft glass Fernbach flasks. A diphtheria strain of low toxicogenicity, the use of other types of glassware, an unfavorable iron concentration or an unfavorable surface/volume ratio are some of the factors which may

decrease this yield. For maximum yields and reproducibility, care must be taken to use clean Pyrex vessels throughout the preparation of the medium.

The medium was allowed to stand at 35°C, for 3 days. The flasks were then inoculated with a Park-Williams No. 8 strain (Albany No. 5 culture), incubated for 6 days at 35°C, after which the organisms were filtered off through paper and the toxin collected through a Berkefeld N candle. The yield varied between 30 and 37 Lf per ml.

Purification of toxic filtrates: Care must be taken, throughout the entire process, to keep the pH above 6. The saturated ammonium sulfate solutions should be adjusted to about pH 7. A typical purification was carried out as follows (2):

15.3 liters of toxic filtrate adjusted to pH 7 to 7.4 with acetic acid (total Lf = 490,000; $Kf_{33} = 17^{****}$) were concentrated to 2300 ml. by distillation *in vacuo* below 35°C. The concentrate was filtered and to the filtrate 1100 gm. of solid ammonium sulfate were added. After the mixture had stood overnight in the cold, the pink precipitate was collected on hardened filter paper, dissolved in distilled water, and made up to 500 ml. (total Lf = 500,000; $Kf_{37} = 12$). 200 ml. of neutralized, saturated ammonium sulfate solution were added and after standing in the cold, the inactive precipitate was filtered off and a further 350 ml. of neutralized saturated ammonium sulfate added. The precipitate which formed contained most of the toxin and was collected by filtration, dissolved in distilled water, and made up to 100 ml. (total Lf = 460,000; $Kf_{46} = 7$). This solution was treated with 45 ml. of ammonium sulfate, filtered, the precipitate discarded, and the filtrate treated with a further 50 ml. The final precipitate designated fraction A by Pappenheimer (2) formed a clear dark red solution when dissolved in water. The overall yield up to this point was about 80 to 90 per cent. The procedure from this stage on was designed to give a product of maximum purity but involved extensive loss of material.

Fraction A was diluted to 825 ml., treated with 165 ml. alumina craem (7) and centrifuged. The precipitate was discarded after washing with distilled water. The supernatant fluid and washing (volume = 1020 ml.) were treated with 1000 ml. saturated ammonium sulfate solution, and the precipitate which formed (fraction

****Kf is taken to represent the flocculation time in minutes at 42 C. The subscript denotes Lf per ml. The flocculation tests were carried out in the usual manner with 1 ml. portions of toxin and varying amounts of antitoxin. The Kf is a sensitive index for damage to the toxin such as is caused by exposure to pH below 6.

B₁) was collected by filtration. An additional 1000 ml. of saturated ammonium sulfate were added to the filtrate and the resulting precipitate (fraction B₂) was collected by filtration.

Fraction B₁ dissolved in 60 ml. of water, was treated with 12 ml. alumina cream. The centrifuged precipitate was washed and discarded. To the supernatant and washings (volume = 150 ml.) 130 ml. saturated ammonium sulfate were added. The precipitate (C₁) was removed by filtration, and an additional 150 ml. saturated ammonium sulfate were added to the filtrate. The precipitate which formed (C₂) was collected by filtration and the filtrate was discarded. Fraction B₂ was treated with alumina cream and fractionated with ammonium sulfate like fraction B₁. The first ammonium sulfate precipitate was combined with C₂, the second one was designated C₃, and the filtrate was discarded.

C₂ was dialyzed against two changes of 0.1 per cent NaHCO₃. 120 ml. of the dialyzed solution were treated with 25 ml. of alumina cream. The precipitate was centrifuged, washed and discarded. The supernatant and washings (165 ml.) were treated with 150 ml. saturated ammonium sulfate, a small precipitate was filtered off and discarded. The filtrate was treated with a further 100 ml. of saturated ammonium sulfate yielding a precipitate (fraction D) which was collected by filtration. The filtrate was discarded.

30 ml. of D were dialyzed overnight in the cold against 2 liters of 0.5 per cent NaHCO₃ solution. The dialyzed solution was made up to 50 ml. and treated with ammonium sulfate. Fraction E precipitated between 45 and 55 ml. of saturated ammonium sulfate, and fraction F was precipitated by addition of another 45 ml. of saturated ammonium sulfate to the filtrate from fraction E. Each of the fractions, D, E, and F was dialyzed against two changes of 0.1 per cent NaHCO₃ solution and then against three changes of distilled water.

Some properties of the partially purified fraction A and of the three most highly purified fractions D, E, and F are listed in table 1. These final fractions formed pale yellow solutions. They gave positive biuret, ninhydrin, xanthoproteic and Millon's reactions in dilute solution. The Hopkins-Cole and Ehrlich's p-dimethylaminobenzaldehyde tests for tryptophane were strongly positive. The material gave only a weak Molisch test in 1 per cent solution and contained no phosphorus. The nitroprusside test for sulfhydryl

TABLE 1

Properties of the Purified Diphtheria Toxin (1,2)

	Fresh Crude Toxin (2)	Pappenheimer's fractions (2)				Eaton's products (1)
		A (partially purified)	D	E	F	
Nitrogen, per cent*			16.1	15.9	15.8	16
Sulfur, per cent*			0.70	0.80	0.75	
Ash, per cent			1.36	1.59	1.35	
$[\alpha]_D$			-36°	-40°	-24°	
Isoelectric point			4.1±0.1			
Nitrogen per Lf, mg.	ca. 0.05	0.0005-0.0006	0.00046	0.00045	0.00046	about 0.0005
M.L.D. per Lf†	40		30	30	30	20-35
Precipitinogen titer ‡	0.2Lf	2.8 Lf	16Lf	16Lf	16Lf	
Kf ₈₀	15		15	16	14	

* Corrected for ash.

† To within about ± 20 per cent.

‡ Ring test against rabbit antiserum to inactive diphtherial protein. The values given are the Lf content of the highest dilution giving a definite ring test after 2 hours at 25°C.

was negative. The toxin was completely precipitated from 1.5 per cent solution between 0.5 and 0.6 saturation with ammonium sulfate. Exposure to pH more acid than 6 was found to result in damage as reflected by increased flocculation time (Kf) and lowered toxicity. The slowly flocculating damaged material was less soluble in one-half saturated ammonium sulfate than was unaltered toxin and could be separated in this manner.

Pappenheimer (2) obtained a small amount of nontoxic diphtheria protein which was precipitated between one-third and two-thirds saturation with ammonium sulfate. Rabbit antisera to this protein were employed to detect non-toxic bacterial protein in purified preparations of diphtheria toxin. Precipitin tests indicated that 98 to 99 per cent of the material originally present in crude toxin was removed during the purification. The absolute amount of non-toxic bacterial protein present cannot be estimated since quantitative precipitin determinations with antiserum to the non-toxic protein were not done (Cf. II, 7, 8).

With antisera obtained by injecting rabbits with washed diphtheria bacilli, Eaton (8) was able to show that bacterial protein con-

stitutes only 0.5 to 2 per cent of the total protein in his most highly purified preparations. Eaton (8) also showed that the red porphyrin compound present in crude toxin and found in small quantity even in highly purified toxin, is not combined with the protein but is adsorbed during the precipitations.

Pappenheimer, Lundgren and Williams (9) and Petermann and Pappenheimer (10) have determined some of the physical properties of purified diphtheria toxin. Between pH 5.6 and 10.1 its sedimentation constant, S_{20} , was 4.6 in 0.5 per cent solution. Below pH 5.6 the toxin was insoluble and at pH 10.8, S_{20} was less than 4.6 indicating some change. S_{20} was found to be independent of concentration between 0.24 and 1.02 per cent. D_{20} was 6.0 with protein concentrations at 0.29 and 0.56 per cent. The molecular weight was calculated to be 74,000 with f/f_0 of 1.22 and the ratio of length to width assuming an unhydrated molecule was 4.7.

Pappenheimer (11) studied the effect of ketene and of formaldehyde on diphtheria toxin. Short acetylation at pH 6 to 7 was found to destroy the toxicity without affecting the ability to combine and flocculate with antitoxin. During the detoxication a number of free amino groups were acetylated. An analogous reduction in amino nitrogen was found after detoxication with low concentrations of formaldehyde in alkaline solution.

Chemical investigations of bacterial toxins have been reviewed by Eaton (12).

Both botulinus (13, 14) and tetanus (15, 16) toxins have recently been prepared in crystalline form. The neurotoxin of the Shiga bacillus has also been partially purified (17). The α -toxin of *Cl. Welchii* has been shown to possess lecithinase activity (18-23); when the toxin was incubated with normal human serum under anaerobic conditions, the medium became opalescent due to the presence of a fat-like material (18). This effect of the α -toxin was specifically inhibited by antitoxin (20). The α -toxin was subsequently found to produce opalescence of egg lecithovitellin (21) and MacFarlane and Knight (22) established that phosphorylcholine and a diglyceride were formed by the action of the α -toxin on lecithin. Zamecnik and Lipman (23) have introduced a manometric method for measurement of the lecithinase activity of a toxin.

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BOIVIN-TYPE ANTIGENS

Many Gram-negative bacteria have been found to contain complex antigens containing carbohydrate, lipid, and a protein or polypeptide-like material. These substances were first isolated by Boivin (1, 2) and have been named after him. The somatic O antigens of the salmonella group (1, 2, 3, 4), the somatic antigens of the dysentery bacillus (5, 6) and antigens from the meningococcus and gonococcus (7), and brucella (8) have been found to be of this general type. The products obtained have shown marked toxicity for mice (1-7), induced leukopenia and fever (9), and the Schwartzman phenomenon (9), and produced severe hemorrhage in tumor-bearing animals at the site of the tumor (10). They were found to be antigenic in animals and in man.

These antigens have been prepared by several different methods. Freeman, Challinor and Wilson (11) have used three of these methods with *Salmonella typhimurium* and *typhosum*. When available for the organism to be studied, synthetic media are to be preferred for growing the organisms, since contamination with other proteins and with agar is avoided. Bacteria are collected by centrifugation of broth cultures in a Sharples centrifuge, washed with alcohol or acetone and ether, and dried.

1. Extraction with trichloroacetic acid (0.1-0.5N): The dried organisms are uniformly suspended in five times their weight of water, cooled to 0° C., and an equal volume of ice-cold 0.5 N trichloroacetic acid is added (0.1 N has been used (11), 0.5 N was required in the case of the meningococcus and gonococcus (7), the original Boivin procedure used 0.25 N.) The mixture is well shaken and kept at 0° C. for three hours. It is then centrifuged at high speed to remove any insoluble bacterial residue and the slightly opalescent solution was dialyzed against running water. Any precipitate formed during dialysis is removed and the solution filtered through an L2 Chamberland candle. The filtrate, which is still opalescent, was concentrated *in vacuo* (ultrafiltration may be used) and the antigen precipitated by the addition of ethyl alcohol to a concentration of 68 per cent (by weight). The precipitate is

washed with alcohol and ether and dried *in vacuo* over phosphorus pentoxide to a white powder.

2. Tryptic digestion: (11, 3, 4): 10 grams of organisms are suspended in 2 liters of distilled water, 100 ml. of toluene added to prevent bacterial and mold growth, and 4.5 ml. N NaOH are added to bring the pH to 8.2-8.5. 100 mg. Fairchild trypsin is then added and the solution incubated at 37° C. After 2.5 days an additional 100 mg. of trypsin is added and digestion continued for a total of 5 days. As digestion proceeds, the pH falls and alkali is added to maintain a pH of 8.2-8.5. The course of the digestion may be followed by removal of samples at various times and determining amino nitrogen (III, 14). Digestion has been found to be maximal after 5 days (11). After digestion the solution is neutralized to pH 7.0 by addition of normal HCl, concentrated to one-half volume *in vacuo* and clarified by centrifugation to give an almost water-clear solution. This solution is then filtered through an L2 Chamberland filter and dialyzed against running water. During dialysis, a large increase in volume occurs and the solution becomes opalescent. It may then be concentrated by ultrafiltration. The supernatant is treated with alcohol to a concentration of 68 per cent by weight, the precipitated antigen centrifuged off, washed with alcohol and ether and dried *in vacuo* over phosphorus pentoxide.

3. Extraction with diethyleneglycol (11, 12, 6): The diethylene glycol should be distilled at 135°-142° C. *in vacuo* at 10 mm. Hg. 50 gm. of dried organisms are suspended in 500 ml. of diethylene glycol, shaken vigorously at 37° C., for 2 hours and then kept at room temperature for 24 hours. The bulk of the bacteria are removed by centrifugation at ordinary speeds and the solution finally clarified by centrifugation at 15,000 r.p.m. (cellophane tubes should not be used, since cellophane is soluble in diethylene glycol) and filtered through a Chamberland L2 filter. The slightly opalescent solution is then dialyzed free from diethylene glycol. During dialysis the solution becomes strongly opalescent. The dialyzed solution is concentrated *in vacuo* and ethyl alcohol is added to a concentration of 68 per cent by weight to precipitate the antigen. The product is washed and dried as above.

The properties of Boivin-type antigens obtained from several microorganisms by the methods outlined above are given in table 1. Rough strains have been found to yield very little of these antigens.

TABLE 1
Properties of Bovine-Type Antigens From Various Bacteria

Organism used	Methods of Preparation	Yield	Ash	N	Reducing sugar on hydrolysis	$[\alpha]$ 5461	P	Medium used	Mouse Lethal Dose	Reference
<i>S. Typhimurium</i>	Trichloroacetic acid	% 3.5	% 9.0	% 2.5	% 48	degrees +47	%	Synthetic	mg. 0.47	(11)
	Tryptic Digestion*	3.9-6.5	6.1-12.5	3.0-6.1	47-51	+75 to +83		Synthetic	0.13-0.27	(11)
	Diethylene glycol	0.5	9.7	2.4	51	+78		Synthetic	0.61	(11)
<i>S. Typhosum</i>	Trichloroacetic acid	3.6	9.1	4.2	36	+90		Synthetic	0.23	(11)
	Tryptic Digestion†	3.4-5.2	12.0-12.5	6.8-7.7	30-35	+93 to +112		Synthetic	0.31	(11)
	Diethylene glycol	0.06	13.7	4.3				Synthetic		(11)
<i>Sh. Paratyphenteriae</i> Type V	Tryptic digestion or Diethylene glycol			4.5	50		1.5	?	0.5	(13)
<i>Sh. Dysenteriae</i> (Shiga)	Diethylene glycol	4.7-7.8		3.8			1.3	Agar	0.1	(6,14)

* Average of six preparations.

† Average of three preparations.

The data on *S. typhimurium* and *S. typhosum* are taken from (11). Other preparations from organisms grown on agar-containing media were found to give tests for agar (11). Very few preparations have been characterized in the ultracentrifuge or by electrophoresis but extensive chemical studies have been carried out (1-7, 11-12). The antigen obtained from *Sh. paradysenteriae* Type V, however, was found to be homogeneous by electrophoresis (13). Quantitative immunochemical studies with these antigens are considered in detail in II, 8 and 9.

Linton, Smith, and Krejci (15) have found that concentrated broth cultures of four strains of typhoid organisms showed three electrophoretic components. Serological reactivity of a concentrate from an O strain of organisms was limited to the slowest migrating component. The middle component of strains containing Vi antigen also showed serological reactivity. Reviews on salmonella (16) and dysentery (17) are recommended.

Goebel, Binkley and Perlman (13) have introduced a method of preparing Boivin-type antigens from *Sh. paradysenteriae* by extraction with aqueous 50 per cent pyridine. The extraction is less specific than that with diethylene glycol since nucleic acid and some immunologically inert polysaccharide are extracted. These may be precipitated by addition of one-half volume of acetone to the pyridine extract.

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CHAPTER 51

PNEUMOCOCCAL TYPE-SPECIFIC POLYSACCHARIDES

Recently, methods (1,2) have been worked out for the isolation of specific polysaccharides from culture filtrates of pneumococci without the use of heat, strong acid, or alkali, thus avoiding degradation (14, 15) caused by the older more drastic techniques (3). In general, these newer procedures involve concentration of the culture filtrate to a convenient volume by vacuum distillation (1) or preferably by ultrafiltration (2), partial separation of the polysaccharide from accompanying impurities by repeated fractional precipitation with alcohol in the presence of sodium acetate and acetic acid (1, 2); removal of protein by shaking with chloroform and butyl alcohol (1) (a modification of the method used by Sevag (4)); and elimination of any glycogen or starch, if present, by methods depending upon the properties of the individual polysaccharides (1). The products are isolated as the neutral sodium salts. These are obtained entirely devoid of color and yield solutions characterized by extraordinarily high viscosity. The purification technique employed by Brown (2) consists mainly of fractional alcohol precipitation, while Heidelberger, Kendall and Scherp (1) obtained somewhat purer products by using the chloroform-butyl alcohol treatment and special methods in addition to fractionation with alcohol. The general procedure given below must be varied slightly for the different polysaccharides and depends on the nature of the impurities present.

PREPARATION OF CULTURE MEDIUM

Meat infusion broth*: Suspend 1 lb. of lean chopped beef in 1 liter of water. After soaking overnight in the icebox, boil the mixture for five minutes, strain through cloth, and filter through paper to remove fat. To the clear filtrate add:

2 gms. NaCl

2 gms. $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$

15 gms. standardized Granular Peptone (Difco)

Bring to boil and adjust the pH to 7.8-8.0 with 2M NaOH, about

* Kindly supplied by Mr. C. M. Soo Hoo, Department of Medicine, College of Physicians and Surgeons.

TABLE I

Analytical Data on Preparations of the Type-specific Polysaccharides of Pneumococci

Type	Kjeldahl N	Amino N	Phos- phorus	Acetyl	Ash	$[\alpha]_D$	η Rel. of 0.10% sol. in 0.9% NaCl at 20°C	Acid equiva- lent	Reduc- ing sugar after hydroly- sis*	Constituents identified
	per cent	per cent	per cent	per cent	per cent	degrees			per cent	
I	4.78—	1.97—	0.19—	6.6—	9.19—	+253—	1.65—			
I(1)	4.84— 4.62— 5.22*	2.10 2.0*	0.32 0.00— 0.25*	6.8 7.1— 10.0*	9.93 0.0— 3.42†	+256 +278— +294*	1.69 1.10— 1.69	590— 650	30	galacturonic acid, nitrogen- ous substance
II	0.26		0.06	1.52	5.46	+ 57	1.76			
II(1)	0.14— 0.73*		0.00*	0.4— 3.8*	0.1— 3.70†	+ 53— + 58	1.04— 1.64	600— 1020	86— 100	glucose, aldo- bionic acid, uronic anhydride
III	0.09—		0.00—	0.32	13.8—	— 36—	2.34—			
III(1)	0.30 0.06— 0.43*		0.01 0.00*	0.5— 0.9*	14.7 0.2— 6.5†	— 37— — 31— — 37	2.79 1.09— 3.14	330— 360	84	glucose, glucu- ronic acid glucose, amino sugar
IV	5.24	0.31	0.49	15.5	5.17	+ 33	1.38			
V	5.01	0.00	0.25	12.7	3.16	— 45	1.06			
VIa	0.32— 0.48		4.03— 4.24	2.1	13.8— 14.1	+112— +114	1.12— 1.20			
Vib ‡	0.32		3.99	3.75	13.4	+113	1.16			
VII	3.19	0.37	1.23	11.1	3.36	+ 71	1.17			
VIII	0.20		0.06— 0.09	0.41	0.73— 7.52	+124— +126	2.44			
VIII(18)	0		0		0	+125		750	76	glucose, glucu- ronic acid
VIII(16)	0.2*			0.05*	3.1†	+123*	2.50			
IX	3.15— 3.26	0.04	0.23— 0.25	9.22	5.28— 7.15	+117	1.28— 1.30	720	87	
X	2.08		2.93	5.06	10.9	+ 22.4	1.12			
XI	1.40— 1.62		2.34— 2.99	15.5— 16.3	8.30— 9.93	+102	1.27— 1.29			
XII	4.69	0.22	0.58	11.1	4.26	+ 3.6	1.25			
XIII	1.62— 1.86		3.07— 3.23	8.23— 8.80	10.50	— 28.4— — 28.8	1.19— 1.26			
XIV	2.41		0.41	6.99	1.28	+ 6.8	1.12			
XIV(19)	1.91— 2.08*		0. 6.6— 7.7*	0.56— 1.57	0.56— 1.57	+ 6.5— + 12.6*				acetyl glucos- amine, galactose
XV	2.46		3.08	9.80	12.2	+ 32.	1.26			
XVI	3.16	0.07	2.81	7.51	9.96	+ 18.4	1.29			
XVII	0.09		2.51	4.28	8.33	+ 4.2	1.28			
XVIII	0.57		3.07	6.52	9.92	+ 76.	1.71			
XIX	3.82	0.16	3.05	7.75	10.7	+ 33	1.09			
XX	1.50— 1.56		2.35— 2.62	10.3— 12.0	7.97— 8.76	+ 4.8— + 7.2	1.19			
XXI	1.20		2.68	7.08	8.82	+ 67.	1.11			
XXII	0.42— 0.69		0.27	4.42— 5.08	4.75— 5.92	+ 95.— + 96.	1.21— 1.25			
XXIII	0.11— 0.27		3.77— 3.79	0.87— 1.44	12.4— 12.7	— 0.4— — 0.8	1.30— 1.65			
XXIV	1.63		3.03	4.79	10.1	— 34.	1.56			
XXV	4.72	0.15	0.87	14.4	5.52	+128	1.09			
XXVII	2.75		3.12	7.02	10.8	+ 53	1.68			
XXVIII	1.59— 1.76		5.99— 6.08	3.87— 4.21	14.2— 14.8	+ 49— + 50	1.67— 1.69			
XXIX	1.53		3.22	5.15	10.5	— 51	1.22			
XXX	1.73		3.06	4.49	10.0	+ 50	1.25			
XXXI	0.90		0.55	7.70	5.80	— 20.4	1.25			
XXXII	1.67		6.38	4.60	16.2	+ 32.	1.45			

All figures are calculated on a dry basis. No allowance is made for ash, except where noted.

Preparations are those of Brown (2) unless otherwise specified.

* Calculated to the ash-free basis. † Ash as sodium. ‡ Originally classified as type XXVI.

14 ml. being required. While hot, pass through fine filter paper. Refilter the first portion of the filtrate. To the clear broth add: 3 grams glucose (1 gm. of glucose if to be used for the seeding culture). Distribute broth in tubes or flasks and sterilize in the autoclave.

For small vessels 15 min. at 15 lbs./sq. in. steam pressure is adequate. Larger vessels require longer periods of time, 25 min. being recommended for 1-1.5 liter amounts.

Peptone dialyzate broth (5) (medium F-26)*: To 1160 grams peptone (Parke Davis or Wilson-Notri) add tap water to make 4.5 kg. and stir to dissolve. Place half in each of two cellophane tubes (about 114 mm. diameter, 840 mm. length, and 0.089 mm. wall-thickness); tie both ends of the tubes securely with twine. Immerse in 24.5 kg. tap water heated to 50-70°C. and adjust the height of the tubes if necessary during dialysis so as to maintain approximate equality of liquid-level inside and outside the tubes. Heat rapidly to 90°C. and maintain at this temperature for seven hours. Remove the tubes and contents, and store the Peptone dialyzate solution at 4°-6°C. overnight.

Dissolve in separate portions of tap water:

5 gms. NaCl

1 gm. K_2HPO_4

1 gm. KH_2PO_4

Combine, add 500 gms. peptone-dialyzate solution, and make up to 1000 gms. with tap water. Adjust pH to 7.7-7.8. Autoclave in bulk for 40 min., add 7.5 gms. of cane sugar, and make up to weight. Filter through paper and cotton. Usually dispense 3200 ml. amounts in 4 liter bottles and autoclave 30 min.; 100 ml. amounts in 200 ml. bottles and autoclave 15 min.

PREPARATION OF CULTURE

10 liters of meat infusion broth or preferably of ultrafiltered peptone broth are inoculated with a highly virulent strain of pneumococcus which has recently been passed through mice to enhance virulence. Heavy growth occurs followed by autolysis. After 72-96 hours at 37° C., 1% phenol is added, the culture allowed to stand overnight at room temperature and tested for sterility. Smears from

* From Wadsworth, A. B. Standard Methods. Williams and Wilkins Co. (Courtesy of Author and Publishers).

individual flasks are examined and any culture found contaminated is discarded. The cultures are then centrifuged in a Sharples centrifuge to remove bacterial debris and the effluent concentrated 10 to 20 fold by vacuum distillation (1) (for technique see (6)) or preferably by ultrafiltration (2) (cf. III-33) through an alundum candle coated with a 4.5 per cent nitrocellulose (Parlodion) membrane which retains the specific polysaccharide. Clogging of the ultrafilter can be minimized by treatment with celite and preliminary passage of the broth through an uncoated alundum candle. The ultrafiltrate is tested for the absence of specific polysaccharide with homologous type-specific antiserum previously absorbed with "C" substance. Any filtrate that gives a precipitin reaction, indicating leakage of the filter, is refiltered through an intact membrane. Ultrafiltration concentrates the polysaccharide 10-20 fold and much nitrogenous material is removed by passage through the filter. After passage of all broth, saline containing 1% phenol is passed through the filter until the effluent is nearly colorless.

The vacuum-evaporated or ultrafiltered concentrate is then centrifuged for several hours at 2-3000 RPM or until it is as clear as possible. The precipitate is washed several times with water in the presence of a little toluene as preservative, until the washings no longer react with specific antiserum.

For 1 liter of concentrate 100 g. of crystalline sodium acetate, $\text{NaAc} \cdot 3\text{H}_2\text{O}$, (the anhydrous salt should not be used because it gives off heat on solution) and 10 ml. of glacial acetic acid are added to over neutralize the alkalinity of sodium acetate and the polysaccharide is precipitated by addition of $1-1\frac{1}{2}$ liters ($1-1\frac{1}{2}$ vols.) of 95% ethyl alcohol to the well-stirred solution. Slightly more alcohol is required in the case of the polysaccharides from types V, XI, XV, XX, XXII and XXXI, S-IV requires the most alcohol (2). The precise amount may be determined rapidly in a preliminary experiment before the main lot is precipitated. Much of the pneumococcus "C" substance remains in solution after addition of $1-1\frac{1}{2}$ volumes of alcohol, but 3-5 volumes are sufficient to precipitate it. For the precipitation of the type-specific carbohydrate excess alcohol should therefore be avoided.

After standing overnight, most of the supernatant, which contains "C" substance, is siphoned off and the precipitate is collected by centrifugation. It is dissolved in about 200 ml. of distilled water.

20 g. sodium acetate and 2 ml. of glacial acetic acid are added and the solution is shaken mechanically for 1-2 hours with about 40 ml. chloroform and 8 ml. of normal butyl alcohol (4). Protein is denatured in the process and an emulsion forms. To separate the mixture, it is centrifuged for $\frac{1}{2}$ to 1 hour at 2000 r.p.m. If protein is present, an opaque white emulsion appears at the interface between the chloroform and the aqueous phase. When much protein is present, the emulsion may incorporate all the chloroform. The aqueous layer is sucked off and the treatment with chloroform and butyl alcohol is repeated until an emulsion is no longer formed after 8-24 hours of shaking and a negative biuret test is obtained. At each step, the emulsion layer is washed with water and the washings combined with the main solution. Enough 95% alcohol is added to precipitate the polysaccharide.

The precipitated polysaccharide is centrifuged and dissolved in water to a volume of about 100 ml. If the solution is very opalescent, alcohol is added until a heavy turbidity is formed (ca. 120-140 ml.); the polysaccharide does not flocculate in the absence of electrolyte. Centrifuge for 10-15 min. at 2000 r.p.m. and discard the precipitate. Add 10 gm. of sodium acetate and 1 ml. of glacial acetic acid. The polysaccharide appears at once as a flocculent precipitate.

After centrifuging and dissolving the polysaccharide in water to about 50 ml. (solution should be quite clear) tests for phosphate with ammonium molybdate solution and for glycogen and starch with dilute iodine solution are performed. If negative, the final precipitation is made with redistilled alcohol, sodium acetate and acetic acid, the precipitate washed twice with redistilled alcohol, filtered on hard paper and dried *in vacuo*, first over CaCl_2 and finally to constant weight over P_2O_5 in high vacuum at room temperature.

Special methods (after 1, 7): SI may be precipitated with Cu^{++} and thus freed from starch or glycogen. For every 100 ml. of SI solution add 10 ml. of saturated copper acetate solution slightly acidified with acetic acid. The light blue Cu-polysaccharide precipitate is centrifuged off, dissolved in 20 ml. of neutralized 10% sodium acetate solution and precipitated with 10 ml. of alcohol. This is repeated until the precipitate is free from copper.

SII is not completely precipitable by copper. Glycogen may be removed by adjusting the solution to pH 6.5 and adding a small amount of saliva (1). After a short time the iodine test becomes neg-

ative. Protein impurities added in the saliva are removed by repeated shaking with chloroform and butyl alcohol. If the polysaccharide solution still contains nitrogen, further purification may be achieved by several precipitations in the cold with 5 volumes of glacial acetic acid in the presence of 5% sodium acetate.

SIII may be freed from nitrogenous contaminants by several precipitations as the barium salt with barium chloride, or as the copper salt with copper acetate. The barium or copper is removed by repeated precipitations from neutralized 20% sodium acetate solution by addition of alcohol.

Miscellaneous techniques: Pneumococcus "C" substance, if present in preparations of type-specific polysaccharide, may be conveniently removed by precipitation of the latter with absolute methyl alcohol. This technique has been successfully employed with type V capsular polysaccharide. Propionic acid in the cold has also been found useful for differential precipitation (7a).

The polysaccharide may be obtained from washed suspensions of intact pneumococci by extraction of protein with 95 per cent phenol. Contamination by broth constituents can thus be avoided (20).

The type-specificity and virulence of pneumococci has been clearly shown to be due to the presence of capsular polysaccharides specific for each type of pneumococcus. These polysaccharides have been obtained from the intact organisms, but are usually prepared from broth in which the polysaccharide has been liberated by autolysis of the bacteria. They have been found to be antigenic in the mouse (8) and in man (9); but fail to produce precipitins in the rabbit (10) unless combined in the intact bacterial cell (11) or with protein by artificial means (12, 13).

The purification of the pneumococcal type-specific polysaccharides provides an excellent example of the need for avoiding drastic treatment during the process of preparation and the value of quantitative immunochemical methods in demonstrating that any alteration in properties has occurred. The original methods for purifying the pneumococcal polysaccharides developed by Heidelberger, Goebel and Avery (3) using qualitative precipitin tests with horse-serum as a guide, involved concentration of broth on a steambath, precipitation with alcohol in acid or alkaline solution. The final products obtained by these methods showed qualitatively by dilution

tests as strong serological activity as did the original broth, but in the case of the type I polysaccharide, Enders noted that type I serum still reacted with broth after removal of the antibody with purified polysaccharide (14). Pappenheimer and Enders (15) purified the type I substance, carefully avoiding the use of alkali, and obtained a material which possessed all the activity of the original broth and was not precipitated by acetic acid. On treatment with alkali some loss of serological activity occurred and the material became precipitable with acetic acid. Avery and Goebel (11) obtained similar results and showed that alkali treatment split off an O-acetyl group the loss of which accounted for the diminished serological reactivity.

Subsequent studies by Heidelberger, Kendall, and Scherp (1) disclosed that antipneumococcal rabbit sera were much more sensitive indicators of alterations in the immunological properties of the type specific polysaccharides than were horse antisera. They observed in types 1, 2, and 3 polysaccharides that different preparations of polysaccharides of the same type, all of which removed the same amount of antibody from horse antiserum, varied considerably in the amount of antibody that each could remove from rabbit antiserum. Further study showed that these differences were due to a partial depolymerization of the polysaccharides when heated in air; in the presence of broth, oxidation also appeared to be involved. Methods avoiding the use of heat, strong acid, and alkali were developed and yielded purified products which precipitated all of the type-specific antipolysaccharide from rabbit antisera (16); these polysaccharides, unlike those prepared with heating, had a high relative viscosity and hence had probably been less depolymerized. The observation that rabbit antisera may be a more sensitive indicator of the degree of degradation of serologically active substances, may be of considerable value in studies with other microorganisms, especially as Heidelberger and Kendall had earlier found that the partial-hydrolysis products of S I and S III precipitated horse antiserum but not rabbit antiserum (17).

Characterization of polysaccharides: Preparation of accurate standard solution: About 200 mg. of the air-dry sodium salt of the polysaccharide are ground to a powder in an agate mortar. The material is transferred to a small, ground glass stoppered weighing bottle and dried at room temperature over P_2O_5 in high vacuum to

constant weight (± 0.1 mg.). Since the polysaccharides are hygroscopic the weighing bottle should be stoppered as soon as the dessicator is opened preliminary to a weighing. When the weight has remained constant for at least two successive days of drying, the material is quantitatively transferred from the weighing bottle to a small beaker, the weighing bottle is again stoppered and reweighed. The weight of the polysaccharide transferred is obtained by difference.

Add a few drops of distilled water (just enough to wet the powder) to the polysaccharide and stir with a small rod until a smooth paste is obtained. Add more water dropwise and stir. After addition of about 2 to 3 ml. of water add a drop of chloroform and place the beaker in the ice box until the next day. This will give the polysaccharide sufficient time to imbibe water and form a uniform solution. The following day the material is quantitatively transferred to a 100 ml. calibrated volumetric flask and made up to volume with water. When samples are removed for analysis pipettes calibrated to contain the volume desired should be employed or the solution should be weighed since aqueous polysaccharide solutions are highly viscous.

Relative viscosity in water (III, 18): Measure the relative viscosity at 20.0°C . of a 0.2% aqueous solution of the polysaccharide with an Ostwald viscometer of 5 ml. capacity. Since the values for the viscosity in water are greatly affected by the presence of small amounts of salt they are less useful for comparison of different preparations than are the values in saline.

Relative viscosity in 0.9% sodium chloride solution: To the aqueous 0.2% polysaccharide solution used above add an equal volume of 1.8% sodium chloride solution. The viscosity of the resulting 0.1% polysaccharide solution in 0.9% sodium chloride is measured at 20.0°C . in a 5 ml. Ostwald viscometer. (See III, 28).

The relative viscosity in saline is a good index of the quality of a polysaccharide preparation. Drastic treatment causing depolymerization will be reflected in a lower viscosity. On the other hand, preparations made from formalinized broth show unusually high viscosities.

Optical rotation: (see III, 32)

Chemical analyses: Weigh out 10 to 30 mg. of vacuum-dry polysaccharide for ash analysis. About 20 to 30 mg. should be used

for nitrogen analysis by the micro-Kjeldahl method. Acid equivalent may be determined as described in (III, 16), acetyl, reducing sugar, and uronic anhydride as given in (III, 15, 17, and 18).

Immunochemical analysis: Quantitative precipitin analysis in the antibody excess zone with a calibrated homologous antiserum is one of the most effective methods for comparing different polysaccharide preparations (II, 8). The maximum amount of antibody nitrogen precipitable by different preparations may also prove of value.

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CHAPTER 52

d (-) GLUTAMYL POLYPEPTIDE

This polypeptide, which was first isolated from encapsulated strains of *B. mesentericus*, *B. anthracis* and *B. subtilis* by Ivanovics and Bruckner (1), is one of the most interesting immunologically reactive substances. It was shown to be a polypeptide composed entirely of d(-) glutamic acid with 40-50 units per molecule. It was reported by Tomcsik and Szongott (2) to fix complement with anti-anthrax sera and 0.01 mg. produced fatal anaphylaxis in a passively sensitized guinea pig. Precipitin reactions of polypeptide with horse and rabbit anthrax and mesentericus antisera have been studied quantitatively by Ivanovics (3). The polypeptide, however, did not induce antibody formation in rabbits.

Since not all strains of *B. mesentericus* and *B. subtilis* produce peptide, care must be taken to select a suitable strain. This can readily be done by growing small tubes or flasks of strains for several days, centrifuging or filtering off the organisms, adjusting the pH to 6.0 and adding 5 per cent by volume of a saturated solution of CuSO_4 . The copper salt of the peptide separates as a fine light green precipitate. Bovarnick (4) found *B. subtilis* strain 41259, received from the Bureau of Plant Industry, U. S. Dept. of Agriculture as No. 712 to be the most satisfactory for peptide production.

For quantitative determination of the amount of peptide present, the precipitate formed by adding 5 per cent by volume of saturated CuSO_4 solution to a measured volume of culture filtrate is centrifuged off, washed twice with dilute CuSO_4 solution, suspended in 5-10 ml. of water and made up to 1.5 N with HCl. Most of the precipitate dissolves and a small amount of reddish insoluble material is removed and discarded. An aliquot portion of this solution is analyzed for nitrogen by the micro-Kjeldahl method. All of the nitrogen may be assumed to be peptide nitrogen and the peptide content of the sample calculated (4).

Sauton's medium has been used by both Bruckner and Ivanovics (5) and by Bovarnick (4). Two gm. of citric acid, 0.05 g. ferric ammonium citrate, 0.5 gm. MgSO_4 (added as 5 ml. of a 10 per cent solution to avoid precipitation), 0.5 gm. K_2HPO_4 , 20 gm. of glycerol and 4 gm. of either l-asparagine or l(+) glutamic acid are dis-

solved in tap water, the pH adjusted to 7.4 with strong ammonia and the solution made up to one liter in a 3 liter Erlenmeyer flask and autoclaved at 121°C. for 15 minutes. Each liter of broth is inoculated with a few ml. of a 15-18 hour culture. The flasks are incubated at 34°C. A pellicle forms in 2-3 days. Bovarnick (4) states that daily shaking to prevent pellicle formation retarded polypeptide production. Peptide production reaches a maximum after about 7 days and then decreases rapidly (4). This decrease may be delayed by addition of larger amounts of glycerol to the medium. After incubation the cultures are filtered, adjusted to pH 6.0, and 5 per cent by volume of saturated copper sulfate solution is added. The precipitate of the copper salt of the polypeptide is centrifuged off, washed twice with dilute CuSO_4 , suspended in water and concentrated HCl is added to make the solution 1.5 *N*. The bulk of the precipitate dissolves and the reddish brown insoluble material is removed and discarded. The HCl solution is dialyzed against 0.5 *M* citrate buffer at pH 5.0 until free from copper and the citrate is then removed by dialysis against distilled water. These preparations contain a considerable amount of ash which may be removed by dialysis for 24 hours against 0.1 *N* HCl (4). The dry peptide may be obtained by evaporating off the water *in vacuo*.

Properties of d(−) glutamyl polypeptide:

N per cent 10

Acid equivalent 160

Electrophoretic mobility (6) -19.5×10^{-5}
cm²/volt. sec. at pH 8.1 in 0.1 μ
phosphate buffer

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CHAPTER 53

BLOOD GROUP SUBSTANCES

Blood group A, B, and O specific substances have been found in a variety of tissues and secretions of animal and human origin. Blood group A substances have been prepared from commercial peptone (1, 2) and hog gastric mucin (3-8) and from the abomasus (fourth stomach) of the cow (9, 10). The need for the selection of individual hog stomach linings was established by the observation (11) that products from pools of hog stomachs showed both blood group A and blood group O specificity and by the demonstration (12) that certain individual hog stomach linings on purification yielded products identical in glucosamine, reducing sugar, acetyl, nitrogen and relatively viscosity with those showing blood group A activity but which were inactive in this respect. Subsequent studies established that individual hog stomachs contained only A substance or only O substance (13, 14) but that some hog stomachs (14), presumably from heterozygous hogs, contained both blood group A and O substances. Horse stomachs have been used as a source of blood group A and B substances (15).

The products from these various sources appear to be closely related serologically to the human blood group A factor. The purest preparations from hog gastric mucin have been shown to be polysaccharide-amino acid complexes (5, 6). Human gastric juice (16) and saliva (16) have also been used for the preparation of blood group substances, but the yields of materials obtained are very small. B and O specific substances have been isolated from gastric juice of individuals of blood groups B and O as well (17). Individuals whose fluids (saliva, gastric juice) contain blood group substances are called secretors, those whose fluids do not contain these substances are called non-secretors (18). The discovery (19) that the fluids obtained from human pseudomucinous ovarian cysts contained much larger amounts of blood group substances than do other human fluids, constitutes a significant advance toward the study of the relationship between human and animal blood group A substances (20).

Morgan and King (6) have used two methods for the preparation of the blood group A substance from hog gastric mucin, one employing fractionation with sodium sulfate and the other extraction with

90 per cent phenol and precipitation with alcohol at a concentration of 10 per cent by volume.

This latter method has proven most advantageous for the preparation of the blood group A and O substances from individual hog stomachs (12). The procedure employed is as follows:

Freshly obtained hog stomach linings cut into small pieces, were allowed to autolyze at 37° C. in 400 ml. of citrate-HCl buffer and toluene at about pH 2.3 for 2 weeks, at which time the pH had risen to 6-7. The residues were filtered off, 5 volumes of ethanol were added to the filtrates, and the stringy white precipitates were filtered, washed and dried.

The dried alcohol precipitate was shaken for 1 day with 10 ml. of 90 per cent phenol; the insoluble portion was removed by centrifugation, suspended in 10 ml. of phenol, and again shaken for 1 day. After centrifugation, the residue was reextracted with 5 ml. of 90 per cent phenol. The combined viscous supernatants were centrifuged until perfectly clear. Occasionally, in some of the preparations, a white lipid material would accumulate at the surface. Most of this lipid could be removed by skimming with a spatula, and the remainder by filtration with gentle suction through a Schleicher and Schull filter paper No. 589-1H. It is essential that the phenol solutions be perfectly clear and free of this lipid or the final products will be contaminated and will not be completely soluble in H₂O or saline. To the clear phenol solution, stirred mechanically, a mixture of equal volumes of ethanol and 90 per cent phenol was slowly added dropwise until the final alcohol concentration reached 10 per cent by volume, and after standing overnight at room temperature, the translucent jelly-like precipitate was separated by centrifugation. This was redissolved in 90 per cent phenol by shaking. The solution was clarified by prolonged centrifugation and then reprecipitated with alcohol-phenol as above. It may be necessary to add a small crystal of sodium acetate to ensure flocculation. The precipitate was finely suspended by rubbing in ethanol and allowed to soak overnight to remove phenol. The white product was washed 3 times with alcohol and with ether and finally dried to constant weight *in vacuo* over P₂O₅. The substances obtained were completely soluble in H₂O or saline. The yields and analytical properties of preparations from 10 individual hog stomach linings and of sample 1A from hog gastric mucin are listed in Table 1; seven of the stomachs showed blood

group A activity, the remaining three were inactive in this respect but have recently been shown to show blood group O activity. The sample from hog 9 exhibited both A and O activity.

TABLE I
Properties of Purified Blood Group A and Inactive (O) Substances Obtained from Individual and Pooled Hog Stomach Linings

Preparation	Method	Activity of original digest	Yield per lining	Ash as Na	N	Reducing sugar as glucose*	Glucosamine†	Glucosamine Ratio Reducing sugar	Glucosamine N Total N	Acetyl	Relative viscosity: 0.2 per cent solution in 0.9 per cent NaCl
			mg.	per cent	per cent	per cent	per cent			per cent	
Hog 1	Peptic digestion	Active	230		6.4	55	32	0.58	0.39		1.52
Hog 2	Peptic digestion	Inactive	600	0.7	5.9	56	32	0.57	0.42	9.4	1.63
Hog 3	Peptic digestion	Active	410	0.4	6.4	58	34	0.58	0.42	10.2	1.65
Hog 4	Peptic digestion	Active	320	0.9	6.5	55	33	0.60	0.40	9.4	1.50
Hog 5	Peptic digestion	Active	980	0.4	6.6	58	34	0.59	0.41	10.0	1.56
Hog 6	Peptic digestion	Inactive	490	0.7	6.1	59	34	0.58	0.44	9.9	1.47
Hog 7	Autolysis	Inactive	310	1.1	5.7	58	32	0.55	0.44	9.3	1.56
Hog 8	Autolysis	Active	380	0.9	5.9	61	34	0.55	0.45	11.3	1.39
Hog 9	Autolysis	Active	400	0.8	6.0	57	33	0.54	0.45	10.2	1.49
Hog 10	Autolysis	Active	660	0.9	6.1	59	34	0.58	0.43	10.7	1.71
1A§				1.0	6.1	56	30	0.54	0.39	9.1	1.38

Values not corrected for ash.

*Reducing values given were obtained by the Hagedorn-Jensen method after hydrolysis for 2 hours with 2N HCl.

†Glucosamine determined after hydrolysis for 2 hours with 2N HCl.

§1A was obtained from Wilson's gastric mucin.

From (12)

The products (6) obtained by these methods from gastric mucin have been found to be rather highly viscous. They were about 90 per cent homogeneous when examined electrophoretically at pH 4.0 in acetate and at pH 8.0 in phosphate buffer of 0.1 ionic strength. At pH 8.0 the mobility was -0.4×10^{-5} cm²/volt-sec. The A substance showed very high activity in inhibiting isoagglutination with human sera containing anti-A and in inhibiting hemolysis of sheep erythrocytes with rabbit anti-A sera. The polysaccharide is composed of N-acetylglucosamine and galactose residues (4-6), but the amino acid moiety has not been identified. The A substance forms an elastic gel in borate buffer at pH 8.5. Degradation by heat results in a marked decrease in viscosity, and in ability to form gels with borate (6); the property of inhibiting sheep cell hemolysis by anti-A rabbit sera, however, is increased (5, 6). Unheated preparations of A substance possess all the "virulence-enhancing" properties of gastric

mucin on bacteria. Recently, Bray, Henry and Stacey (21) have demonstrated that l-fucose is also present in blood group A substance. This has been confirmed (14) and l-fucose has been shown to be a constituent of both the A and O substances from hog stomach.

Fluids from pseudomucinous cysts of secretors of blood group A were studied by Morgan and Van Heyningen (19). Fluids were lyophilized. Several samples had isoagglutinin inhibiting potencies equal to the best purified substances obtained from gastric mucin and many times more active than the most potent A saliva. Activity of these human materials was associated with the fraction insoluble in 90 per cent phenol and fractions prepared in this manner were several times more active than the A substance from gastric mucin. Cyst fluids from secretors of blood groups B and O also contained large amounts of B and O substances. Heterologous isoagglutinins were also found in cyst fluids.

The blood group A substance was found not to be antigenic in rabbits (6), but by combination with the conjugated protein of the Shiga dysentery antigen, a complete antigen was obtained (21). In humans, however, the blood group substances are complete antigens and Witebsky, Klendshoj and McNeil (22) found that intravenous injection of purified A and B substances into humans lacking these antigens produced considerable increases in isoagglutinin titer.

Addition of A and B substances to O blood has successfully reduced the isoagglutinin titer prior to use as universal blood in transfusion (23-25).

Purified blood group A substance obtained from hog gastric mucin or stomach linings and A and B substances from horse stomachs have been found to give precipitin reactions with sera containing homologous isoagglutinins. The micro quantitative precipitin method has been used to measure the amounts of these isoagglutinins quantitatively (7). Sera of individuals immunized with purified A and B substances showed increases in precipitable antibody nitrogen proportional to the increases in titer and in capacity to neutralize A or B substance. The quantitative precipitin curves obtained by adding increasing amounts of blood group A substance to a given volume of serum from immunized individuals of groups O or B were typical of other antigen-antibody systems (1, 2).

Little is known of the chemical nature of the Rh factor. The

recent demonstration by Witebsky (27) that the Rh factor occurs in amniotic fluid offers considerable promise for its early isolation and characterization. In a recent report partial concentration of the Rh factor from erythrocytes was obtained; the material, however, contained the A and B substances as well (28).

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APPENDIX

A. CENTRIFUGES AND CENTRIFUGATION

A refrigerated centrifuge such as the portable model PR-1 manufactured by International Equipment Co., Boston, is indispensable for immunochemical work. A cold centrifuge may be improvised by surrounding a regular room temperature centrifuge with a tightly wound copper refrigeration coil with an internal spiral lamination and an insulating jacket. Alternatively, an ordinary centrifuge may be placed in a cold room or into a commercial ice-cream freezer. To improve cooling through better circulation of air, two or three holes are drilled into the cover of the centrifuge.

Angle centrifuges are unsatisfactory for quantitative precipitin and agglutinin analysis since soft specific precipitates or agglutinated bacteria are deposited as a streak along the wall of the tube and particles are often lost on decanting the supernatant.

Breakage of 250 ml. centrifuge bottles during centrifugation can be reduced by placing two rubber bands around each bottle, one near the top and another near the bottom. The free space between the bottle and the metal carrier should be partly filled with water to relieve the centrifugal strain on the glass. Loss of valuable material as a result of breakage of glass tubes in the centrifuge can be avoided by placing the glass tube inside a slightly larger plastic tube. For centrifugation at high speed (above 2500-3000 r.p.m.), it is preferable to use plastic tubes such as those manufactured by Lusteroid Corporation. In contrast to glass tubes, plastic tubes require closely fitted individual metal carriers to avoid distortion. Various numbers of 8 ml. and 15 ml. conical Pyrex centrifuge tubes and 13 x 100 mm. Wassermann tubes, used in quantitative precipitin tests may be centrifuged in the large metal carriers with safety.

The acceleration a , in cm. per sec., imparted by a centrifuge of radius r , in cm., and speed n , in revolutions per minute, is given by the equation:

$$a = \frac{4 \pi^2}{3600} \cdot r \cdot n^2 = 0.0109 \cdot r \cdot n^2 \quad [1]$$

Expressed in terms of the acceleration of the earth's gravitational field (981 cm/sec.²), the acceleration equals

$$a = \frac{0.0109 \cdot r \cdot n^2}{981} = 1.11 \times 10^{-5} \cdot r \cdot n^2 \quad [2]$$

For example, if a centrifuge of 15 cm. radius runs at 2000 r.p.m., it exerts an acceleration 670 times that of the earth's gravitational field. It is evident from equations [1] and [2] that a precise description of a centrifugal operation should include, in addition to the time of centrifugation, the radius as well as the speed of the centrifuge, or should be given in terms of the acceleration.

In balancing the load of a centrifuge, it is important not only to place equal weights on opposite sides but also to have the same weight distribution in respect to distance from the center of the centrifuge. A thin-wall centrifuge bottle should therefore not be counter-balanced with a heavy-wall bottle even if the difference in weight is made up with water.

B. COLORIMETRY (cf. III, 30)

If a beam of monochromatic light is passed through a colored solution, light is absorbed according to the relation

$$\log \frac{I_0}{I} = K c l \quad [3]$$

in which I_0 and I are respectively the intensities of the light before and after passage through the colored solution, K is a constant dependent on the wavelength of the light and the absorption characteristics of the colored material, c represents the concentration, and l denotes the length of the light path through the solution. The term $\log I_0/I$ is known as the optical density or extinction, D , i. e.

$$D = K c l \quad [4]$$

The fraction I/I_0 is termed the transmittance, T . Therefore,

$$\log T = - K c l \quad [5]$$

With instruments like the Duboscq colorimeter, the comparison between unknown and standard solutions of the dye is made by varying the light path, l , through the unknown until an exact match between unknown and standard is obtained, that is, until the transmitted light intensities are the same. Since the incident light intensity is also the same, it follows from equation [3] that

$$c_1 l_1 = c_2 l_2 \quad [6]$$

where the subscripts 1 and 2 refer to the unknown and the standard, respectively. The concentration, c_1 of the dye in the unknown is therefore calculated as follows:

$$c_1 = \frac{c_2 l_2}{l_1} \quad [7]$$

Modern photoelectric colorimeters or spectrophotometers operate with a constant light path and are designed to give an absolute measurement of color intensity in terms of the transmittance T or the optical density D . For purposes of calculation, the latter is more convenient since D is a direct, linear function of the concentration of dye if the light path is constant, i.e.,

$$D = K'c \quad [8]$$

The value of K' for a given color is found from a series of determinations on solutions of known concentration and is used in subsequent analyses to calculate c from D . It is not necessary to include standards in every set of analyses. If it is found in the standardization of a color reaction that equation [8] is not obeyed, a calibration curve should be constructed by plotting D against c . The wave length of the light used for analysis of a given color is generally chosen in the range of maximum light absorption. With spectrophotometers equipped with a prism or diffraction grating, it is advisable to verify the wavelength calibration occasionally. It is also recommended to check the calibration of the transmittance or optical density scales by means of a colored solution or glass filter of known T or D .

C. BUFFERS, INDICATORS AND MEASUREMENTS OF pH (cf. III, 31)

Buffer solutions generally consist of weak acids (or bases) which have been partly neutralized, so that both undissociated acid, HA , and anions, A^- , are present in appreciable concentration. The pH of a buffer may be calculated *approximately* by means of the equation

$$pH = pK + \log \frac{C_{A^{-*}}}{C_{HA}} \quad [9]$$

in which the term C_{A^-} represents the concentration of anion and C_{HA}

* Activity coefficient should be employed.

denotes the concentration of undissociated acid. The term pK is defined as the negative logarithm of the dissociation constant of the acid, i.e., $pK = \log \frac{1}{K}$, just as pH equals the negative logarithm of the concentration, or more precisely, of the activity of hydrogen ion. When an acid is one-half neutralized $CA^- = CHA$ and consequently $pH = pK$. Therefore, a mixture of equimolar parts of acetic acid ($pK = 4.7$) and sodium acetate has a pH of 4.7. The same pH would be obtained by adding $\frac{1}{2}$ mol of sodium hydroxide to 1 mol of acetic acid.

Primary buffers: The following buffers have been recommended as standard buffer solutions of known pH and may be used for checking pH meters, etc. (1). They should be made up accurately with reagents dried to constant weight.

0.05 molal Phthalate buffer (1): 10.211 g. acid potassium phthalate is dissolved in one kilogram of water.

pH at $20^\circ C$ = 4.001

pH at $25^\circ C$ = 4.008

pH at $30^\circ C$ = 4.015

Phosphate-NaCl buffer (1): 0.3530 g. NaH_2PO_4 + 0.6390 g. Na_2HPO_4 + 0.172 g. $NaCl$ is dissolved in one kilogram of water.

pH at $20^\circ C$ = 7.218

pH at $25^\circ C$ = 7.202

pH at $30^\circ C$ = 7.195

Sodium dihydrogen phosphate and disodium hydrogen phosphate should be dried slightly above room temperature for a day or two before completely dehydrating them to constant weight at $110^\circ C$. to prevent fusion of the hydrated salts. These solids may be checked for purity by phosphorus analysis.

Buffer solutions for general use: The pH of all solutions should be measured, since small variations in pH usually occur with different lots of reagents, etc. 0.1 ionic strength buffers for electrophoresis (cf. III, 14). Indicated mixtures to be made up to one liter:

pH

4.1	350 ml <i>M</i> acetic acid	+	100 ml. <i>M</i> Sodium acetate
4.2	300 ml <i>M</i> acetic acid	+	100 ml. <i>M</i> Sodium acetate
4.6	100 ml <i>M</i> acetic acid	+	100 ml <i>M</i> Sodium acetate
4.8	80 ml <i>M</i> acetic acid	+	100 ml <i>M</i> Sodium acetate
5.0	50 ml <i>M</i> acetic acid	+	100 ml <i>M</i> Sodium acetate

- 5.3 12 ml $M/5$ Na_2HPO_4 + 460 ml $M/5$ NaH_2PO_4
 6.0 50 ml $M/5$ Na_2HPO_4 + 350 ml $M/5$ NaH_2PO_4
 7.0 125 ml $M/5$ Na_2HPO_4 + 125 ml $M/5$ NaH_2PO_4
 8.0 163.5 ml $M/5$ Na_2HPO_4 + 10 ml $M/5$ NaH_2PO_4
 9 50 ml M NH_3 + 100 ml M NH_4Cl
 10 500 ml. M NH_3 + 100 ml M NH_4Cl
 11 110 ml. $M/5$ Na_2HPO_4 + 28 ml. $M/5$ Na_3PO_4
 12 17 ml. $M/5$ Na_2HPO_4 + 75 ml. $M/5$ Na_3PO_4

For combined immunochemical and electrophoretic studies, buffers containing 0.15 M NaCl + 0.02 M phosphate or 0.02 M acetate at the desired pH have been found satisfactory.

Since indicators act as buffers, they should be used in minute amounts so as not to affect the pH of the solution to be tested. The pK of an indicator, as the pK of any weak acid, is the pH at which the indicator is one-half neutralized i.e., it represents the midpoint of the color change.

Several common indicators are listed below. For more complete information, see W. M. Clark, The Determination of Hydrogen Ions, The Williams and Wilkins Company, Baltimore, Maryland.

Indicator	pK	Color		pH Range
	(approximate)	acid	alkali	(approximate)
brom-cresol-green	4.7	yellow	blue	3.8— 5.4
methyl red	5.1	red	yellow	4.2— 6.3
phenol red	7.7	yellow	red	6.8— 8.4
phenolphthalein	9.7	colorless	red	8.3—10.0
thymolphthalein		colorless	blue	9.3—10.5

Alcoholic solutions are generally used. If alcohol is undesirable, aqueous solutions may be prepared by converting the indicator into the salt form.

For precise determinations, glass electrode pH meters have come into general use. For details, see Dole, The Glass Electrode,

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D. CLEANING OF EQUIPMENT

As a general rule, all glassware in contact with protein solutions is washed with water and dilute alkali immediately after use and filled with, or immersed in, dilute alkali until the time for the final cleaning. Never allow dirty equipment to dry. General laboratory glassware such as beakers, funnels, Erlenmeyer flasks, etc. are cleaned with hot soap solution or calgonite, rinsed liberally in tap water and finally several times in distilled water. Allow to dry in air or in an oven. Place in a dust-free cabinet.

Glassware for volumetric measurements (e.g. pipettes, burettes, volumetric flasks and cylinders, etc.) as well as tubes for quantitative precipitin or agglutination tests, and complement fixation tests, are cleaned by immersion in, or by filling with sulfuric acid-dichromate cleaning mixture for at least 10 hours. (Less time will suffice if the cleaning mixture is hot.) Great caution is necessary in handling cleaning solution since it can cause serious injury. Goggles or plastic visors should be worn. All vessels should be inspected for cracks before filling with cleaning solution. Large vessels containing cleaning solution should be kept in an enamel or lead tray or pot. If cleaning mixture is spilled on the skin or clothing, wash at once with copious amounts of cold water.

After use of cleaning mixture, glassware is washed copiously with cold water (hot water may contain grease) and finally twice with distilled water. Test tubes for quantitative serological analysis and serological pipettes are dried in an oven. Package the dry tubes or pipettes in lots of 50 to 100 in sterilizing paper, which will keep out dust and dirt. These packages may be sterilized by dry heat (180°C for 2 hours). Accurate volumetric glassware should not be dried by heat. Volumetric pipettes are dried by drawing a little acetone (reagent grade) through them, followed by a current of air. Volumetric flasks are allowed to dry inverted.

Properly cleaned glassware should not show droplets on its surface when wet with water. This is essential for accurate measurements with volumetric apparatus. It is also important in tubes employed for quantitative serological measurements.

Rubber goods: Caps, stoppers and tubing are flushed with water and soaked in dilute alkali (e.g. 0.01 N) preliminary to washing with soap or calgonite. Gum rubber stoppers and tubing used in drawing blood are boiled in dilute alkali for 5-10 min. and

then in distilled water for another 10-15 min. Rubber goods should be stored in the dark to prevent deterioration.

Syringes and hypodermic needles: These are rinsed with water and dilute alkali immediately after use, then washed with soap or calgonite, rinsed with water and alcohol and dried. For use in skin tests, special care in cleaning syringes and needles is advisable. A cleaning agent is chosen which will destroy the antigen employed. For example, syringes and needles used for tuberculin may be cleaned by boiling in a solution of trisodium phosphate.

The clean and dry syringes are wrapped individually in wrapping paper and sterilized by dry heat. Needles are placed individually in cotton-plugged culture tubes for sterilization by dry heat. A constriction in the middle of each tube will prevent the tip of the needle from striking the bottom of the tube.

For use, the needle is placed on the syringe by inserting the tip of the syringe into the tube holding the needle, and inverting the tube so that the needle falls onto the tip of the syringe. After withdrawal, the needle is fastened with forceps.

E. CALIBRATION OF VOLUMETRIC GLASSWARE

In the metric system the unit of volume is the liter which is defined as the volume occupied by 1 kilogram of water at 4°C., the temperature of maximum density. The usual procedure of volumetric calibration involves determining the weight, W , of water or mercury required to fill the apparatus at a definite temperature, t . The volume then equals W/D , where D is the density of water or mercury at temperature t (for tables of values cf. Handbook of Chemistry and Physics, Chemical Rubber Publishing Co.).

The capacity of volumetric glassware is subject to temperature change due to expansion of the glass, but this effect is so small that it can generally be neglected, unless the temperature deviates markedly from 20°C., the usual standard temperature of calibration. Between 0° and 40°C., the limits likely to be encountered in immunochemical work, errors due to this source are negligible.

Variations in volume of the solution itself with change in temperature are considerably larger. For example, with water, or dilute aqueous solutions, the volume increases about 0.2 part per 1000 for a rise of 1°C. in the vicinity of room temperature. When a high degree

of accuracy is required, as in density experiments (III-23), temperature control is necessary.

In the calibration of accurate volumetric glassware in terms of the weight of fluid contained or delivered, a slight error arises due to the effect of buoyancy of the air on the weight of the fluid. When water is weighed with brass weights, this correction equals about 1 part per thousand, that is, 1 gram of water weighed *in vacuo* has a weight of about 999 mg. in air. This effect may be taken into account by correcting the density of water *in vacuo*, as listed in reference tables, to that in air. For example, the value of D at 20°C . is corrected from 0.9982 *in vacuo* to 0.9972 gm/ml. in air.

Before calibration, glassware is treated with dichromate-sulfuric acid cleaning mixture for several hours. It is then rinsed with water, followed by acetone, and is dried by passage of a stream of clean air. (Do not heat). Properly cleaned glassware will be covered with an unbroken film of water when wet.

Pipettes may be calibrated to "deliver," to "contain," or to "blow-out." While chemists generally employ "deliver" pipettes, in immunochemical work "blow-out" pipettes are preferable. In calibrating the same technique of delivery should be followed as will be employed in use.

Procedure for "blow-out" pipettes: Draw freshly boiled distilled water at room temperature into the pipette above the mark. Wipe the tip and let the level fall until the bottom of the meniscus is tangent to the mark (the pipette should be held vertically, and care should be exercised to avoid parallax). Touch the tip against the wall of the water reservoir and let the contents of the pipette run into a previously weighed flask. Allow to drain and blow out the last drop several times, holding the tip against the wall of the flask. Stopper the flask and weigh. Note the temperature of the water. If the weight delivered from the existing mark is incorrect, make a new mark with a sharpened wax pencil. Determine the weight of water delivered from this new mark and estimate the position of the correct mark from the difference between the first and second marks. Paste a strip of paper around the pipette so that the edge corresponds to the position of the correct mark. Check the delivery from this mark and, if correct, coat the stem of the pipette with paraffin, cut a circular mark through the wax along the edge of the paper, using a piece of strong thread or a knife, and etch with

hydrofluoric acid. Identify the correct mark with an arrow. The permissible error is about 2 parts per thousand for pipettes between 1 and 10 ml. capacity.

Calibration of flasks: Weigh the clean and dry flask. Fill to the mark with water (bottom of meniscus tangent to the mark). Make sure that no drops adhere to the wall above the mark. Weigh the flask and note the temperature of the water. If the mark is incorrect, locate the correct mark as described for pipettes. Alternatively, a flask can be calibrated by delivering the correct volume into the flask from an accurately calibrated pipette.

Calibration of burettes: Burettes should be calibrated in convenient intervals covering the entire range. For example, with a 5 ml burette, weigh the water delivered from 0 to 1 ml, 1 to 2 ml, 2 to 3 ml, etc. Plot the algebraic sum of the errors against the volume delivered, and use the resulting curve for correction of readings.

F. PREPARATION OF ANTIGENS FOR IMMUNIZATION AND INJECTION SCHEDULES

Soluble antigens are generally sterilized by passage through a Chamberland, Berkefeld or Seitz filter. Suspensions of insoluble antigens may be sterilized by treatment with 0.2 per cent formalin for several days in the cold or with 0.3 to 0.5 per cent phenol. The antigen is then centrifuged off, washed once with sterile saline and suspended in saline. Phenol need not be removed. As a preservative for soluble as well as insoluble antigens 1 per cent by volume of a 1 per cent solution of merthiolate is added.

Soluble proteins may be precipitated with alum to obtain an enhanced antibody response. To 100 ml. sterile solution of the protein containing 1.5 mg. of protein per ml., is added 5 ml. of 1 per cent sterile alum. Neutralize with $N/10$ NaOH to maximum turbidity.

There is great variation in the injection schedules followed by different workers. In the laboratories of the authors a course of immunization for rabbits generally consists of 16 intravenous injections administered at the rate of 4 per week, using a total of from 10 to 100 mg. of protein per animal. A typical schedule used with a suspension of alum-precipitated serum albumin containing 1.5 mg. protein per ml., is as follows: 3 injections of 1 ml.; 3 injections of 1.5 ml.; 4 injections of 2ml.; 4 injections of 3 ml.; 2 injections of 5 ml. The first injection of each course is usually given intraperiton-

McFarland Scale No.	ml. 1% BaCl ₂	ml. 1% H ₂ SO ₄
1	1	99
2	2	98
3	3	97
4	4	96
5	5	95
6	6	94
7	7	93
8	8	92
9	9	91
10	10	90

(1) McFarland, J.: J.A.M.A., 1907, **49**: 1176.

eally and subcutaneously. The animals are usually bled on the fifth to seventh day after the final injection. A second course of injections following a similar schedule may be started after a rest period of 1 to 3 weeks.

A similar type of injection schedule may be used for bacterial suspensions. With pneumococci, a total of 0.5-5 mg. bacterial N may be administered in such a course, starting with initial injections of about 0.05 mg. bacterial N.

A convenient procedure for preparing antigens when it is desired to use the adjuvant technic of Freund and McDermott (cf I, 5) is as follows: The desired amount of antigen (i.e. 500 mg. of crystalline egg albumin or 9-10 grams of brain tissue) is dissolved or suspended in 10 ml. of saline containing 1 per cent of phenol as a preservative. Ten ml. of "aquaphor" at the lowest temperature at which it will remain molten are added and the mixture homogenized either with a mortar and pestle or in a Waring blender. Twenty ml. of paraffin oil are then added in which 50 mg. of dried heat-killed tubercle bacilli have been uniformly suspended and the entire mixture homogenized, transferred to a sterile bottle on which a self-sealing rubber cap is placed. For injection 19 to 21 gauge needles are used. Filling of syringes will be facilitated if the emulsion is warmed at 37° C. before use. A convenient injection schedule consists of three injections of

one ml. each at weekly intervals. Injections may be given intramuscularly or subcutaneously.

McFarland turbidity scale (1): This series of standards is readily prepared and has been widely used by bacteriologists and immunologists to specify turbidity of bacterial suspensions or colloid particles for agglutination tests: 1 per cent solutions of barium sulfate and sulfuric acid are prepared. The standards consist of mixtures in the following proportions:

If portions of each mixture are uniformly suspended and sealed in test tubes, they provide permanent standards for use in a comparator.

G. INJECTION OF ANIMALS

In general, sterile technique should be used for injections. Solutions or suspensions should be kept in bottles with self-sealing rubber caps. In filling syringes, the cap through which the needle is to be inserted is swabbed with 70 per cent alcohol. An amount of air equivalent to the volume of solution desired is first drawn into the syringe, and the needle is then inserted through the rubber cap, while holding the bottle inverted. A portion of the air is introduced into the bottle and a portion of the fluid withdrawn by pulling out the plunger. A second portion of air is then admitted into the bottle and solution again withdrawn. This procedure is continued until the syringe contains the desired amount of fluid and is free of air bubbles. The needle and syringe are then pulled out of the cap and set aside with the tip on a piece of cotton or gauze moistened with alcohol.

Rabbits. For intravenous injection, the ear veins are generally used. If a series of injections are to be given, it is advisable to start as close to the tip of the ear as possible and gradually work down toward the base. In this way if the vein becomes obstructed at the site of injection, the same vein may still be used.

The rabbit is placed in a specially constructed box through which its neck and head project and the box is stood on end so that stasis of blood in the head will distend the ear veins. The hair over the portion of the ear to be used is shaved, the ear moistened with 70 per cent alcohol and the needle introduced into the vein bevel upward. The needle is then held against the rabbit's ear with the left hand while the desired amount of solution is injected with the right hand. The syringe is then removed and bleeding stopped by pressure with

a piece of cotton. Twenty-four to 26 gauge needles are used for intravenous injections.

For intracutaneous, subcutaneous or intraperitoneal injections, it is generally advisable to have the rabbit held by an assistant. Subcutaneous injections may be performed satisfactorily alone if the operator has a table containing the syringe and materials at his side. The operator, wearing a rubber apron, places the rabbit facing outward so that the animal's hind quarters are firmly held between the operator's thighs. The skin of the back or sides may then be clipped or shaved and the injection carried out. For intracutaneous injections, use 24 gauge needles, for subcutaneous or intraperitoneal injections, 21 gauge are recommended. Intracutaneous injections are carried out so that a bleb is formed under the skin at the site of the injection. The needle, bevel upward, should be introduced at a very slight angle so as barely to penetrate between the two layers of skin. Subcutaneous injections are performed by pulling up the skin with one hand and inserting the needle into the raised area. For intraperitoneal injections, the needle is inserted at a moderate angle directly into the abdominal cavity and then retracted slightly. Extremely sharp needles should not be used to avoid penetrating the gut.

Guinea Pigs: For intravenous injection of guinea pigs, an assistant is required to hold the animals. The position in which they are held is very important for successful results. The guinea pig has several good veins which can be used with precision after some practice. The best veins are on the upper surface of each hind foot between the outer and middle toes. The animal is held loosely around the chest with the thumb and forefinger of one hand. With the other hand the assistant extends the leg to be injected by holding it by the thigh with the thumb and forefinger. The other hind leg is kept out of the way below the assistant's hand. If the left hind leg is to be injected, it is held with the assistant's right hand, while the left hand is around the animal's chest. The converse arrangement is used for injecting the right hind leg. The animal is held with its leg at a convenient height facing the operator. The operator grasps the extended foot in his left hand and shaves off the hair on the upper surface. The assistant distends the vein by pressure on the thigh. The operator, using a syringe with a sharp 26 gauge needle, introduces the needle into the skin directly between the middle and outer

toe, adjusts the angle of the needle so that it is parallel to the vein and introduces it into the vein. He then holds the base of the needle against the guinea pig's foot firmly with his left hand, the assistant relaxes the pressure on the thigh and the solution is injected. The needle is then withdrawn and pressure applied until bleeding stops. In general, a guinea pig's vein may be used only once until the scar heals. With some guinea pigs, the vein runs on the outside surface of the hind foot just above the footpad. It can be brought into position if the assistant changes the angle at which the pig is held.

There is also a suitable vein in each forearm. The pig is held so that the outer surface of the animal's forearm is presented to the operator, while the vein is distended by pressure. The skin is shaved and the vein may be seen running diagonally across the arm. The skin is stretched taut as the vein is movable and the needle should enter the skin on a line with the vein.

Subcutaneous, intracutaneous and intraperitoneal injections are carried out as described for rabbits with the aid of an assistant.

Mice: Mice may be injected intravenously in the lateral tail veins, of which there are four. The mouse is picked up by the tail so that only the animal's forefeet rest on a coarse wire mesh. The tail is then held between the thumb and the bent forefinger so that a strip of tail rests on the forefinger of the operator. Injections are made by pushing the needle through the skin and then into the vein. A 1 ml. tuberculin syringe with a 1" 25 or 26 gauge needle is used.

For intraperitoneal injection, the mouse is held by the tail with the right hand and a fold of skin at the back of the neck is grasped firmly with thumb and forefinger of the left hand. The mouse is then raised while keeping the tail taut and the animal is turned so that the abdomen faces the operator. The tail is then placed between the palm and fourth and fifth fingers so that the mouse is then held entirely with the left hand. The right hind leg is extended and held together with the tail leaving the right hand free to inject. Intraperitoneal injections are carried out as for rabbits.

Chickens: Small chicks may be held by the wing with their legs resting on the table. Intravenous injections of large chickens require an assistant who grasps the animal firmly by both legs with one hand, while preventing its wings from flapping with the other, removes it from the cage, and places it on its side. The operator takes

the wing and after plucking the feathers, injects into the veins occurring along the under surface.

H. BLEEDING OF ANIMALS AND COLLECTION OF SERUM

Rabbits: Amounts of non-sterile blood up to 5 ml. can be obtained from the marginal ear vein. Place the animal in a rabbit box and stand it on end. Shave the ear and rub it briskly with a wad of cotton wet with 70 per cent alcohol. Place the ear over a lighted 15 watt electric bulb to warm it and make a small longitudinal cut in the ear vein by means of a razor blade. Catch the blood in the test tube. If a clot forms over the cut, wipe it away. Stop bleeding by removing the light bulb and pressing a piece of cotton on the area of the cut.

Amounts of blood up to about 20 ml. may be drawn with sterile precautions from the central artery of the ear. Use a 19 gauge needle and a 20 ml. syringe. Place the animal in a rabbit box and stand the box on end. Rub the ear briskly with alcohol, or preferably with xylol. Insert the needle into the artery with the tip pointing toward the base of the ear.

As much as 50 ml. of blood may be taken by heart puncture, with ether anesthesia, without undue danger to the animal. This requires the aid of an assistant who holds the animal while sitting on a low stool (about as high as an ordinary chair). Pick up the animal by the skin of the back and place its hind legs securely between the thighs of the assistant. Let the rabbit recline in the assistant's lap. The assistant should hold the animal's forelegs in his hands while spreading them apart so that the animal's chest is exposed. Locate the sternum and cut the hair over an area of about one square inch to the right and slightly above the sternum, and apply alcohol or tincture of iodine. Use an 18 gauge needle and a 50 ml. syringe. The operator locates the sternum and the point at which the heart beat is most distinct with the thumb and index finger of the left hand and inserts the needle at a point of the shaved area forming an equilateral triangle with thumb and index finger. Pass the needle through the space between two ribs and then push further and gently toward the position of the index finger until the heart is felt beating against the point of the needle. If it appears to be directly ahead in the direction of the needle, give it a short jab forward to enter the heart. Draw up to 50 ml. of blood, being careful not to move the

move the needle sideways while it is in the heart. Then withdraw the needle rapidly while maintaining its direction. If the heart is not located directly ahead of the needle on the initial insertion withdraw the needle slightly, and change the direction of the needle toward the area from which the heart beat was felt during the initial trial. Instead of a syringe a blood collection set constructed as follows may be used: Fit a 250 ml. centrifuge bottle with a 2-hole rubber stopper holding two small glass tubes, one of which should project about $\frac{1}{2}$ inch into the bottle. Connect a $1\frac{1}{2}$ to 2 foot length of gum rubber tubing to the long glass inlet tube. Place a needle and needle adaptor on the other end of the rubber tube. The short glass tube on the bottle is closed with absorbent cotton to avoid bacterial contamination. The needle is protected during sterilization by a small test tube held in place over it with a rubber band. For sterilization in the autoclave, the entire assembly is wrapped in a towel and placed in a tin can inserted in a tightly woven cloth bag which can be closed with a string at the top. Care should be taken not to kink the rubber tubing so that steam may enter freely during sterilization. If unclotted blood is desired, a 3.8 per cent solution of sodium citrate is placed in the vessel before sterilization. Use 20 to 25 ml. of citrate solution for 100 ml. of blood.

Other animals: Guinea pigs are bled by heart puncture with a 19 gauge needle using the technique described for rabbits. It is advantageous to tie the animal to a board. From a 2 lb. guinea pig up to 15 ml. of blood can be taken without ill effects to the animal.

Small dogs, young pigs and other small mammals may also be bled by the same procedure.

Sheep are bled from the jugular vein with a 13 to 15 gauge needle. The animal is placed into a corner and an assistant holds the animal's head up. The hair is clipped from the neck and a long rubber tube is placed around the lower part of the neck under slight pressure to cause venous stasis. After application of tincture of iodine and alcohol, the needle, pointing toward the head, is inserted into the vein.

Chickens: Chickens are most easily bled from the jugular vein. An assistant holds the chicken by the bony portion of the head with one hand while stretching the bird taut by the legs with the other. The animal rests on a table with its right side upward. The feathers are plucked, exposing the jugular vein which may then be distended

and held in place by slight pressure of the operator's left hand at the base of the neck. Eighteen to twenty-two gauge needles are used and the needle is inserted pointing toward the head of the animal.

Separation of serum: If sterility is essential, the blood is collected in a centrifuge tube or bottle. Otherwise, a petri dish is preferable. The vessel is allowed to stand undisturbed at room temperature until the blood is clotted. Using a sterile pipette or glass rod, the clot is then separated from the walls of the vessel, but if the blood is in a dish, it is better to leave a small area attached to the rim. In addition, the clot may be cut into several slices. Then place the blood in the icebox until the next day so that the clot will contract. The expressed serum is then drawn off and centrifuged to remove any cells. If the blood is contained in a centrifuge tube or bottle, it is also centrifuged before taking off the serum. Withdrawal of serum is conveniently accomplished with a suction tube, or a pipette with a rubber tube attached for suction by mouth. For storage, 1 per cent by volume of a 1 per cent solution of merthiolate is added to the serum as a preservative, and a sterile rubber stopper is placed on the bottle to prevent evaporation. As an additional precaution, the serum may be sterilized by passage through a Chamberland L2 or a Seitz filter.

If the use of preservatives is objectionable, the serum may be stored safely in a deep-freezer or in the dry state after lyophilization.

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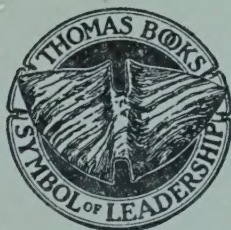
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